Unravelling MAP4K4 Function in Medulloblastoma Cell Dissemination

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UNRAVELLING MAP4K4 FUNCTION IN MEDULLOBLASTOMA CELL DISSEMINATION

A DOCTORAL THESIS

Submitted by
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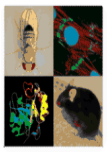

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Zusammenfassung

Medulloblastom (MB), der häufigste bösartige pädiatrische Hirntumor, umfasst vier molekular und genetisch verschiedene Untergruppen von embryonalen Hirntumoren, die sich im Kleinhirn entwickeln. MB betrifft vor allem Säuglinge und Kinder und zeichnet sich durch ein hohes Risiko einer metastatischen Verbreitung aus, welcher die Infiltration von Tumorzellen in das Hirngewebe vorausgeht. Die molekularen Mechanismen, welche die MB-Tumorzellinfiltration kontrollieren, sind zurzeit noch unklar und es existiert bisher keine spezifische Therapie, welche die Verbreitung der Tumorzellen unterbindet. Aktuelle Behandlungsstrategien beim MB, insbesondere die kraniospinale Strahlentherapie angewandt zur Verhinderung der metastatischen Verbreitung, können langfristige Nebenwirkungen für das sich entwickelnde Zentralnervensystem der Patienten verursachen. Daher sind Therapien, welche die Tumorzellinfiltration und die metastatische Verbreitung spezifisch verhindern dringend nötig, da diese die therapeutische Wirksamkeit erhöhen und langfristige Nebenwirkungen verringern könnten.

Die Krebszellverbreitung ist stark mit einer abweichenden oder überhöhten Kinaseaktivität assoziiert. In der vorliegenden Studie haben wir die Ser/Thr Kinase MAP4K4 als ein Molekül im MB identifiziert, welches massgeblich zur Zellmigration beiträgt. Mittels immunhistochemischer Analysen von Tumorgewebe und quantitativen PCR Methoden haben wir eine erhöhte Expression von MAP4K4 in einer Reihe von primären MB Proben im Vergleich zum normalen Cerebellum festgestellt. Dies deutet darauf hin, dass die MAP4K4 das invasive Potenzial der MB Tumorzellen erhöhen könnte.

Die c-Met Rezeptor Tyrosinkinase ist ein Protoonkogen, welche in vielen soliden Tumoren einschliesslich dem MB in erhöhter Menge vorliegt und zur Tumorzellverbreitung beiträgt. Unter Verwendung eines zellbasierten dreidimensionalen Zellmotilitätsassays, kombiniert mit lebend-Zell Mikroskopie, fanden wir, dass der c-Met Ligand HGF die MB Zellmotilität stark erhöht. Dies ist insbesondere in denjenigen MB-Zelllinien der Fall, in denen viel c-Met vorliegt. Wir konnten aufzeigen, dass MAP4K4 die c-Met-abhängige invasive Zellmigration im MB vermittelt und zwar mittels der Kontrolle des F-Aktin Zellskelettes in Lamellipodien, den Strukturen, welche für die Migration und Invasion der MB Zellen erforderlich sind.

Ionisierende Photonenstrahlen-Therapie ist eine gängige therapeutische Modalität für MB. Allerdings fanden mehrere Untersuchungen, dass Photonenstrahlung die Tumorzellmigration verstärken kann. Da der Beitrag der Photonenstrahlung zur MB Zellverbreitung umstritten ist, untersuchten wir die Auswirkungen von Photonenstrahlung auf das Migrations- und Invasionsverhalten der SHH-MB Zelllinien DAOY und UW228. Wir fanden, dass Photonenstrahlung von 1 bis 2 Gray die Zellmigration und die Invasion in beiden Linien erhöht. Interessanterweise war dieser Effekt der Photonenstrahlung in den MB Zellen stark reduziert, in denen wir die MAP4K4 gezielt eliminiert hatten. Obwohl diese Erkenntnisse die Relevanz der MAP4K4 im Rahmen der MB Zellverbreitung weiter hervorhoben, blieben die diesem Vorgang zugrunde liegenden molekularen Mechanismen nach wie vor unklar.

Die Stimulation von MB Zellen mit HGF löst sehr dynamische Membranausstülpungen aus und erhöht die endozytotische Aktivität, zwei Prozesse, die ebenfalls mit der Tumorzellmigration einhergehen. Wir haben daher die Implikation der MAP4K4 in der HGF-induzierter Endozytose und dem vesikulären Transport untersucht. Beide Funktionen hängen von der dynamischen Reorganisation des F-Aktin-Zellskeletts ab. Wir entdeckten, dass die MAP4K4 nicht nur die HGF-induzierte Makropinozytose, sondern auch die Internalisierung des c-Met-Rezeptors und die Aktivierung dessen Effektors ERK beschleunigt. Wir wiesen ferner nach, dass die von HGF angeregte Migration und Invasion von MB Zellen vom Adhäsionsrezeptor Integrin β1 (ITGB1) abhängt und mit einer erhöhten ITGB1-Aktivierung in Lamellipodien der MB-Zellen korreliert. Wir zeigten, dass die beeinträchtigte Migration und Invasion in MB Zellen mit verminderter MAP4K4 Expression nicht nur eng mit der verminderten ITGB1-Endozytose korreliert, sondern auch mit der Reduktion der Menge aktiven ITGB1 in den Lamellipodien.

Zusammengefasst identifizierte diese Studie eine prominente regulatorische Rolle von MAP4K4 im Zusammenhang mit der Verbreitung von MB Tumorzellen. Sie wies nach, dass MAP4K4 die Signale von Wachstumsfaktorrezeptoren auf die Regulation des Aktin Zellskelett überträgt, sowie die endozytotische

Aktivität der Tumorzellen steuert. Dies bedeutet, dass MAP4K4 ein vielversprechendes therapeutisches Ziel darstellen könnte, welches zur Behandlung der Wachstumsfaktor-induzierten Verbreitung von MB Tumoren weiter untersucht werden sollte. Darüber hinaus identifizierte unsere Studie die wachstumsfaktorinduzierte, erhöhte endozytische Aktivität und räumliche Kontrolle der Adhäsionsrezeptoraktivierung als neuartige, möglicherweise therapierbare Mechanismen in der MB Tumorzelle. Wir zeigen, dass diese durch die proto-onkogene Kinase MAP4K4 vermittelt werden und vermuten, dass MAP4K4 dadurch mithilft einen invasiven und bestrahlungsbeständigen Phänotyp in MB Tumorzellen zu etablieren.

Summary

Medulloblastoma (MB), the most common malignant pediatric brain tumor, comprises four molecularly and genetically distinct subgroups of embryonal brain tumors that develop in the cerebellum. MB mostly affects infants and children and is characterized by a high risk of metastatic dissemination, which is preceded by the infiltration of tumor cells into the brain tissue. The molecular mechanisms that control MB tumor cell infiltration are only poorly understood and no specific anti-dissemination therapy exists so far. Current MB treatment strategies, in particular the craniospinal radiotherapy to prevent metastatic outgrowth, can cause long-term side effects for the developing central nervous system of the patients. Therefore, therapies that target tumor cell infiltration and metastatic dissemination specifically, are urgently needed to increase efficaciousness of therapy and to decrease late-treatment complications.

Cancer cell dissemination is strongly associated with aberrant kinase signaling. In the present study, we have identified the Ser/Thr kinase MAP4K4 as a pro-migratory kinase in MB. Using tumor tissue micro array, immunohistochemistry analyses and quantitative PCR methods, we have revealed increased expression of MAP4K4 in a number of primary MB samples compared to normal cerebellum, suggesting that MAP4K4 could increase the invasive potential of the MB tumor cells by promoting cell dissemination.

A well-known promoter of cell dissemination is the proto-oncogene receptor tyrosine kinase c-Met, which is aberrantly expressed in many human tumors including MB. Using a cell-based three-dimensional cell motility assay combined with live-cell imaging, we found that the c-Met ligand HGF induces cell dissemination in MB, especially in cell lines expressing increased c-Met levels. Additionally, we identified MAP4K4 as a novel driver of c-Met-dependent invasive cell dissemination in MB. This increased invasive motility was attributed to MAP4K4 control of F-actin dynamics in lamellipodia, structures required for migration and invasion.

Ionizing photon radiation (IR) therapy is a common therapeutic modality for MB. However, several reports found that IR can increase tumor cell migration. Since IR contribution to MB cell dissemination still remains controversial, we investigated its effects on migration and invasion in the SHH MB cell lines DAOY and UW228. We found that IR increases cell migration and invasion in both lines and that these pro-migratory effects of IR were greatly reduced when MAP4K4 was depleted. Although these findings further underscored the relevance of MAP4K4 in the context of MB cell dissemination, the underlying molecular mechanisms remained unclear.

We found that HGF stimulation of MB cells triggers membrane ruffling and enhances endocytic activity, processes that are associated with tumor cell migration. We have therefore examined MAP4K4 implication in HGF-induced endocytosis and vesicle trafficking, two processes depending on dynamic reorganization of the F-actin cytoskeleton. We found that MAP4K4 not only promoted HGF-induced macropinocytosis but also accelerated c-Met receptor internalization and the activation of its downstream effector ERK. We furthermore found that HGF-driven migration and invasion of MB cells depend on the adhesion receptor Integrin β1 (ITGB1), and are associated with increased ITGB1 activation in lamellipodia of invading MB cells. We demonstrated that impaired migration and invasion after MAP4K4 depletion closely correlated with disrupted ITGB1 endocytosis and reduced levels of activated ITGB1 in lamellipodia of collagen-invading MB cells.

In summary, our findings identified a prominent regulatory role of MAP4K4 towards MB tumor cell dissemination, and revealed that it couples growth factor signaling to actin cytoskeleton regulation and endocytic activity. This implies that MAP4K4 could present a promising novel target to be further evaluated for treating growth factor-induced dissemination of MB tumors. Furthermore, we discovered that enhanced endocytic activity and the spatial control of adhesion receptor activation and turnover through the proto-oncogenic kinase MAP4K4 is a novel, potentially druggable mechanism, which we suspect to sustain an invasive and IR-resistant phenotype in MB.

1. Introduction

1.1 Cancer

Cancer is a genetic disease that is caused by the accumulation of mutations and epigenetic alterations, which ultimately lead to the growth of a mutant clone. The cells within the entity of the mutant clone present uncontrolled proliferation/cell division rates, which subsequently give rise to the formation of a tumor or neoplasm. Provided that the neoplastic cells have not yet become invasive, the tumor is defined as benign. For the majority of such tumors, surgical resection or/and local irradiation of the mass leads to the cure of the patient (Weinberg 1983; Hanahan and Weinberg 2011; Hainaut and Plymoth 2013).

However, a tumor is considered to be cancerous provided that it is malignant, that is when the cells have acquired the capability to invade the surrounding tissue. Invasiveness is an essential characteristic of cancer cells since it allows them to escape from the local tumor, enter the bloodstream or lymphatic vessels, and form secondary tumors called metastases at other sites of the body (Fidler 2003) (Fig. 1).

Malignant tumors are defined according to their tissue of origin. Carcinomas are cancers arising from epithelial cells and represent the most common human cancers. Carcinomas account for about 80% of cases probably due to the fact that epithelial cells present the highest proliferation rates. In addition, epithelial cells are the most likely to be exposed to various forms of physical and chemical damage that favor cancer development.

Sarcomas originate from connective tissue or muscle cells, whereas cancers that do not belong in either of these two broad categories include leukemias and lymphomas, derived from white blood cells and their progenitors (hemopoietic cells), as well as cancers derived from cells of the nervous system.

In parallel with the set of names for malignant tumors, there is a corresponding nomenclature for benign tumors. For instance, an adenoma, vs. a benign epithelial tumor and the corresponding type of malignant tumor is an adenocarcinoma. Similarly, a chondroma and a chondrosarcoma are, respectively, benign and malignant cartilage tumors (Weinberg 2014).

Cancers originating from different cell types consist distinct diseases. Basal-cell skin carcinomas, for example, are only locally invasive and rarely metastatic, whereas melanomas are highly invasive and often form metastases. Basal-cell carcinomas are usually cured following surgery or local irradiation, whereas malignant melanomas, once they have metastasized, are usually fatal (Fidler 1999; Hainaut and Plymoth 2013; Weinberg 2014).

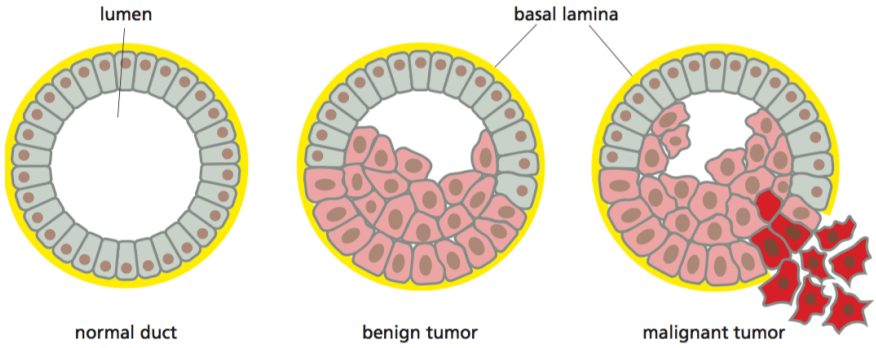

Figure 1: Benign versus malignant tumors: A benign glandular tumor (*pink cells*) remains inside the basal lamina *(yellow)* that marks the boundary of a normal duct structure. In contrast, a malignant glandular tumor (*red* cells) derives from a benign tumor cells that have acquired invasive characteristics (Alberts, Johnson et al. 2015).

1.1.1 Incidence

Estimates of the worldwide cancer incidence and mortality for 2012 reported 14.1 million new cases and 8.2 million deaths caused by cancer. Notably, the most frequently diagnosed malignancies were lung (1.82 million), breast (1.67 million) and colorectal (1.36 million), whereas the most common causes of

cancer death were lung cancer (1.6 million deaths), liver cancer (745,000 deaths) and stomach cancer (723,000 deaths) (Fig. 2) (Ferlay, Soerjomataram et al. 2015).

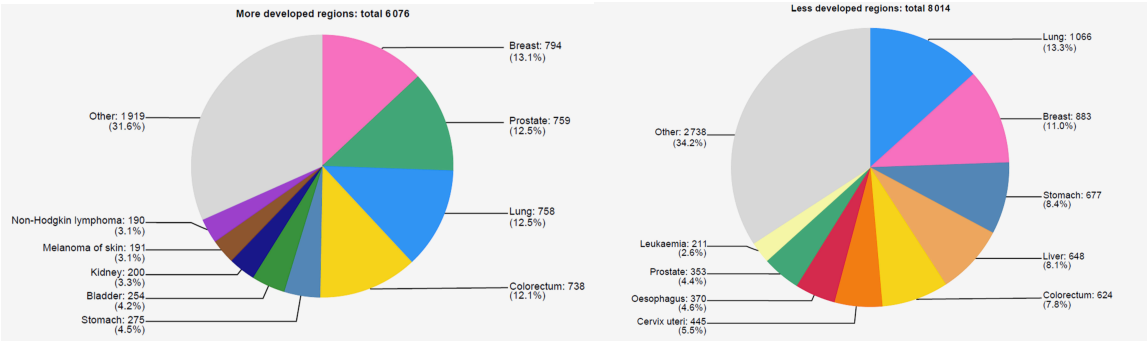

Figure 2: Estimated global numbers of new cases (thousands) with proportions for (a) more developed and (b) less developed regions worldwide, both sexes combined in 2012. The area of the pie is proportional to the number of new cases (Ferlay, Soerjomataram et al. 2015).

1.1.2 Pediatric cancers

Although cure rates for childhood cancers are higher compared to those for adult malignancies, cancer remains the leading cause of disease-caused death among children older than one year of age in developed countries. Interestingly, the spectrum of cancers occurring in the pediatric patients is considerably different from that seen in adults. For instance, the major brain and solid tumors that arise in children, including medulloblastoma, neuroblastoma, rhabdomyosarcoma, Ewing sarcoma and osteosarcoma, are exceedingly rare in adults (Fig. 3). This marked difference in the spectrum of cancers is not unexpected since the majority of pediatric cancers arise within developing tissues during early organ formation, growth and maturation, and are not strongly linked to lifestyle or environmental risk factors. With some exceptions, childhood cancers tend to respond better to certain treatments such as chemotherapy, whereas children (especially very young children) are more likely to be affected by radiation therapy. The unique biology of these developing tissues suggests that the spectrum of mutations that lead to malignant transformation will also differ between pediatric and adult cancers (Siegel, Naishadham et al. 2012).

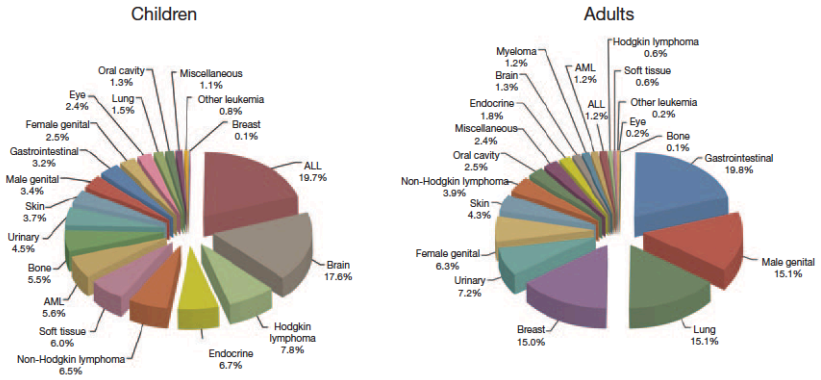

Figure 3: The frequency of cancer types in children (left) and adults (right) on the basis of 2012. Each chart is organized with cancers listed from the most common to the least common in a clockwise fashion (Downing, Wilson et al. 2012).

1.2 Embryonal brain tumors

Embryonal brain tumors consist of a group of biologically heterogenous neuroepithelial tumors that comprise 3.3% of all malignant brain tumors and represent 15.7% and 4.3% of brain tumors diagnosed in patients between 0-14 and 15-19 years of age respectively (Bernstein, Sethi et al. 2013). Based on the histopathological features and genetic aberrations, the World Health Organization (WHO) 2016 classification has grouped the embryonal brain tumors into the following groups (Louis, Perry et al. 2016):

- Medulloblastomas
- ETMR (Embryonal tumors with multilayered rosettes)
- ETMR NOS (not otherwise specified).
- Medulloepithelioma.
- CNS neuroblastoma.
- CNS ganglioneuroblastoma.
- CNS embryonal tumor NOS.
- CNS AT/RT (atypical teratoid/rhabdoid tumor).
- CNS embryonal tumor with rhabdoid features.
- Medulloblastomas (MBs)

1.2.1 Incidence

Medulloblastomas (MBs) arise in the cerebellum (Fig. 4 & 5) and are the most common malignant pediatric brain tumors, accounting for 15-20% of all pediatric CNS tumors (Pizer, Donachie et al. 2011). Most of the cases occur in children aged up to 14 years old (6.8 per million), whereas the frequency decreases in the adult population (0.4-1% of all adult tumors). The median age at presentation of MB is 5-7 years of age and there is a slight male preponderance (40%) (Pui, Gajjar et al. 2011; Siegel, Miller et al. 2015).

1.2.2 Diagnosis

Since MBs are posterior fossa tumors, a frequent symptom of MBs is hydrocephalus which is caused due to the compression of the fourth ventricle, with a consequent increase in intracranial pressure. Other common symptoms are vertigo, vomiting, ataxia and headache. Patients with a tumor localized in the mid-cerebellum may have symptoms that are related to compression of the cranial nerves (nystagmus, diplopia, hearing loss, seventh cranial palsies). On MRI, the appearance of MBs is variable. Typically, MB appears as a round, slightly lobulated, and T1-iso-/hypointense mass (Fig. 5) (Koeller and Rushing 2003).

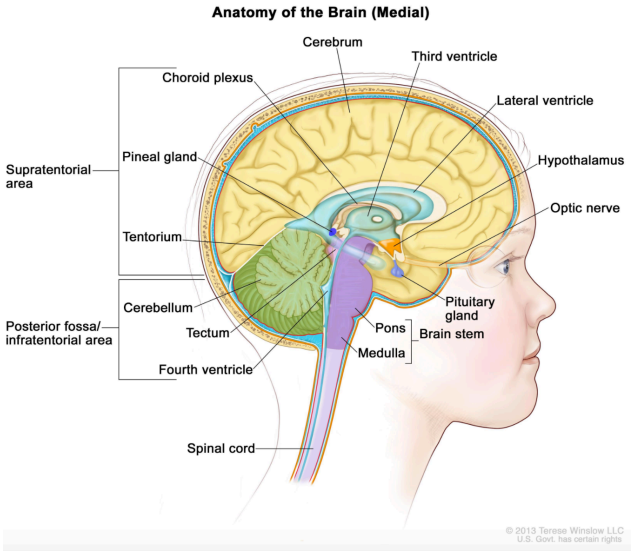

Figure 4. Anatomy of the inside of the brain, showing the pineal and pituitary glands, optic nerve, ventricles (with cerebrospinal fluid shown in blue), and other parts of the brain. The posterior fossa is the region below the tentorium, which separates the cortex from the cerebellum and essentially denotes the region containing the brain stem, cerebellum, and fourth ventricle (https://www.cancer.gov).

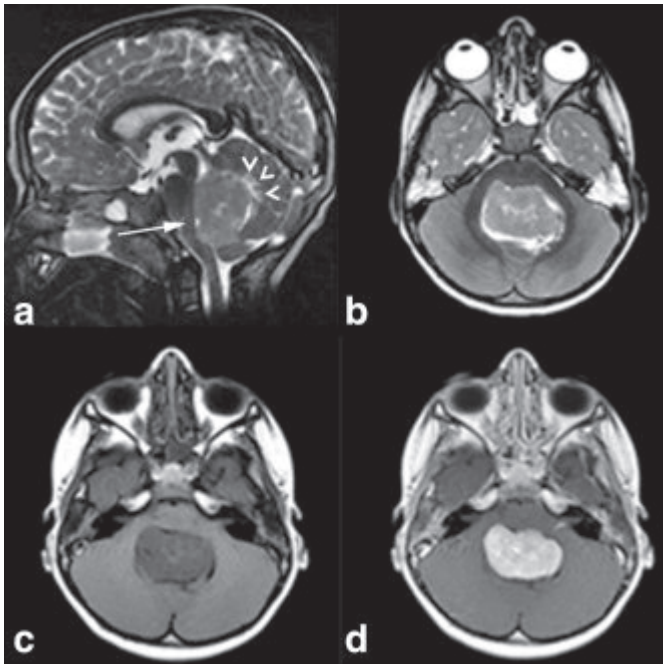

Figure 5: Medulloblastoma. Sagittal and axial T2-weighted (a,b) and axial pre- and postcontrast T1-weighted MR images (c,d) of a 6-year-old boy with classical medulloblastoma. The mass lesion is located in the midline dorsally to the fourth ventricle and pushes the fourth ventricle anteriorly (arrow on a). The tumor is moderately T2-hyperintense to the cerebellar and brainstem gray matter and shows a strong contrast enhancement. Mild perifocal T2-hyperintense edema is seen within the vermis, dorsally to the tumor (white arrowheads on a) (Journal of Magnetic Resonance Imaging Volume 35, Issue 1, pages 32-47, 11 OCT 2011 DOI: 10.1002/jmri.22722 http://onlinelibrary.wiley.com/doi/10.1002/jmri.22722/full#fig4).

1.2.3 Survival

Survival rates depend on the age of the presenting patients, as well as on the applied treatment strategies. Interestingly, 5-year survival is better for the age group 5-14 years (67%), compared to the age group of 1-4 years (47%) (Kohler, Ward et al. 2011). Furthermore, it has been reported that treatment care entailing surgical resection followed by craniospinal irradiation and chemotherapy, results in overall cure

rates of 70-75% for patients older than 3-5 years (Gatta, Botta et al. 2014).

1.2.4 MB Histology

According to the latest WHO classification of CNS tumors, MB tumors are classified into the following histological subtypes based on histopathological features (Louis, Perry et al. 2016):

- Classic MB
- Desmoplastic/Nodular MB
- MB with extensive nodularity (MBEN)
- Large cell anaplastic (LC/A) MB

Among the above histological variants, large-cell anaplastic MBs occur with a frequency of 10-22%. Desmoplastic/Nodular MB and MBEN comprise approximately 7% and 3% of all MBs respectively, while classic MBs constitute the remainder (Fig. 6) (Gilbertson and Ellison 2008).

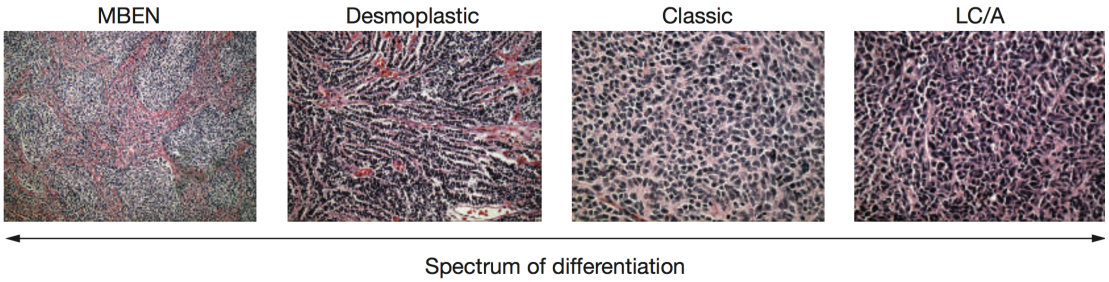

Figure 6: Histopathologic subtypes of medulloblastoma: The histopathologic subtypes of medulloblastoma exist along a spectrum of differentiation and have been investigated for prognostic value, with some studies suggesting an improved prognosis for MBEN and desmoplastic subtypes and other studies showing a worse prognosis for LC/A. (Polkinghorn and Tarbell 2007).

Classic MB is composed of densely packed cells with round-to-oval hyperchromatic nuclei surrounded by scanty cytoplasm. Desmoplastic/nodular MB is a variant that contains nodular, reticulin-free zones, or “pale islands” which represent zones of neuronal maturation. Classic MB exhibits a reduced nuclear to cytoplasmic ratio, a fibrillary matrix, and uniform cells with a neurocytic appearance. These nodules are surrounded by densely packed mitotically active cells, which produce a dense intercellular reticulin-positive network of fibers (Fig. 6).

MB with extensive nodularity (MBEN) occurs mainly in infants and is associated with a good prognosis. It differs from the related nodular/desmoplastic variant by having an expanded lobular architecture (Fig. 6).

The LC/A MB is composed of monomorphic cells with large, round, vesicular nuclei, prominent nucleoli and variably abundant eosinophilic cytoplasm. Groups or sheets of these “large cells” tend to mix with cells that have a different morphology characterized by marked nuclear pleomorphism and nuclear moulding. The latter phenotype has been labelled as “anaplastic” (Fig. 6).

Histologic subtypes are associated with different outcome and thus have prognostic relevance for the patients. Clinical data have indicated favourable prognosis for the nodular/desmoplastic MB, whereas significantly worse prognosis has been evident for the LC/A variant (Massimino, Antonelli et al. 2013; Massimino, Casanova et al. 2013).

1.2.5 Staging

Traditionally, MB patients are stratified into standard risk and high-risk groups according to age, residual tumor and metastatic disease at diagnosis. High-risk patients include those younger than 3 years of age or those having a residual tumor (>1.5 cm^2) or disseminated disease at diagnosis and/or large cells/anaplastic histology (Packer, Rood et al. 2003). In fact, one third of the patients will have metastasis

at the time of diagnosis and two thirds will have leptomeningeal spread by the time of relapse (MacDonald 2008). Moreover, dissemination is the leading cause of treatment failure and the most powerful factor associated with poor survival (Zeltzer, Boyett et al. 1999). Therefore, correct staging is crucial for a reliable risk stratification of patients and is increasingly relevant for the selection of the appropriate therapeutic approach.

Metastatic spread has higher incidence in children than adults (13% vs. 8%) (Frost, Laperriere et al. 1995) and typically follows CSF and leptomeningeal dissemination along the spinal cord. The cerebellum and brain are the most common sites of metastases in conjunction with spinal diffusion. The time interval between diagnosis of the primary tumor and detection of metastases is usually shorter in children than adults (20 months vs. 36 months) (Brandes, Franceschi et al. 2009)

1.2.6 MB treatment strategies

The treatment for standard risk patients includes surgery, craniospinal irradiation and chemotherapy with reported 5-year overall survival of 80%. For patients with metastatic disease, the intensified treatment regimens including high-dose chemotherapy with autologous stem-cell transplantation and non-conventional radiotherapy, have improved prognosis and 5-year overall survival rates between 50 and 70% (Gajjar, Chintagumpala et al. 2006; Sanders, Onar et al. 2008). Among survivors, a major concern is the long-term sequelae caused by treatments which include endocrinological, neurocognitive, behavioral, motor and sensory deficits (Armstrong, Liu et al. 2009). Therefore, there is a critical need to identify new molecular targets that improve tumor growth suppression while minimizing the therapy side effects.

Surgical resection

Surgical resection is the standard therapeutic approach for MB and since it is of high relevance of the outcome, maximum resection is usually aimed for. However, common complications include hemorrhage, meningitis, cervical instability and posterior fossa syndrome (swallowing difficulties, mutism, truncal ataxia and emotional instability) (Albright, Wisoff et al. 1996).

Radiotherapy (RT)

Photon radiotherapy remains the main treatment modality for MB patients older than three years old, with an onset of 4-6 weeks postoperatively and a maximum duration of 50 days [33,34]. Given the propensity of MB to disseminate via the CSF, current standard photon radiotherapy practices across the world involve the use of craniospinal irradiation with a boost to the posterior fossa/primary tumor area, which has raised the five-year progression-free (PFS) and overall survival (OS) rates from 0% to 50–60% in children older than 3 years. By then, a further increase of survival rates by 10–15% was achieved by the addition of maintenance chemotherapy (Fossati, Ricardi et al. 2009).

Proton beam radiotherapy has evolved into an efficient but less toxic therapeutic modality for MB. The advantage of proton beam radiation therapy mainly comes from the difference in depth dose distribution as compared to standard photon beam radiation therapy. Furthermore, proton doses are lower in the normal tissues proximal to the target, reducing thus damage in the healthy tissue adjacent to the tumor. Using normal tissue complication probabilities to predict IQ deficits, proton therapy is expected to decrease the neuropsychological morbidity associated with photon irradiation, especially in children between 4-8 years of age. Proton therapy may also have the potential to decrease the incidence of treatment-induced secondary cancers (Mountford and Temperton 1992), (Bussiere and Adams 2003).

Chemotherapy (CT)

Reduced dose of craniospinal irradiation is usually combined with chemotherapy provided in a

weekly concurrent single-drug (vincristine) dose followed by a multidrug regimen that can include cisplatin, lomustine, or cyclophosphamide. Based on this regimen, five-year event-free survival (EFS) is over 80% (Packer 1990).

Favorable results with five-year progression-free survival and overall survival rates of more than 80% have been obtained using a total craniospinal radiation dose of 23.4 Gy and a boost to the posterior fossa up to 54–55.8 Gy (with concomitant vincristine), followed by maintenance chemotherapy with cisplatin, vincristine, and either lomustine or cyclophosphamide (Lannering, Rutkowski et al. 2012; Packer, Zhou et al. 2013).

Late sequelae

Although current treatment advances have significantly improved survival rates of MB patients, it is known that a high proportion of survivors eventually suffer from significant sequelae, including cognitive decline, impairment of growth and endocrine function, hearing loss, and secondary neoplasms, neuropsychological and behavioral deficits, which lead to significant impairment of quality of survival.

Awareness of these sequelae is required not only for the choice of an adequate tumor treatment regimen, but also during aftercare. Quality of survival can be optimized by adequate information of the patient and his/her family, by detection and treatment of any sequelae, and by minimization of their repercussions on the daily life. Clearly, the development of novel treatment concepts, such as treatment de-escalation, alternative radiotherapy modalities and schedules (e.g. proton therapy), and incorporating of targeted agents into the treatment protocols aim towards the improvement of the quality of survival (Kiehna, Mulhern et al. 2006).

Neurocognitive impairment is particularly important. Due to the variety of contributing factors (e.g. anatomical site of tissue destruction, radiation field and dose, chemotherapy regimens, and patient-related factors such as age or still little understood traits), the cognitive outcome of brain tumor patients is highly individual and only predictable to a certain degree. Although no homogeneous neuropsychological profile exists in MB patients, the most affected cognitive areas are mental processing speed, fine motor skills, working memory and concentration, with the magnitude of the effects often increasing over time (Palmer, Reddick et al. 2007; Duffner 2010).

Behavioral and psychological outcomes of MB patients are also often impaired. Internalizing problems, higher global distress and depression are mentioned in the literature (Zebrack, Gurney et al. 2004). Other common sequelae include neurological deficits (e.g. cerebellar dysfunction, posterior fossa syndrome) caused by tumor growth or by surgery, (Puget, Boddaert et al. 2009) sensorineural hearing deficits due to radiotherapy and ototoxic chemotherapy, and endocrinologic deficiencies due to damage of the hypothalamo-pituitary axis by irradiation (i.e. growth hormone deficiency, precocious or delayed puberty, hypothyroidism, hypocortisolism) (Packer, Gajjar et al. 2006).

Considering the severity of the aforementioned treatment-related side effects for MB patients, the development of new alternative therapies that target MB dissemination while circumventing therapy-induced morbidity are required.

1.2.7 Biology

1.2.7.1 MB Subgroups

One of the most important advances in the increasing knowledge of MB is the discovery of molecular patterns allowing the division of MBs into distinct molecular subgroups with different cytogenetic, mutational and gene expression signatures, as well as different demographics, histology and prognosis. According to the above integrated genomic studies, MB is not considered as a single morphologically homogenous entity, but is in fact comprised of several different diseases at the molecular level. Large scale studies deciphering the transcriptional landscape of MB have revealed the following

distinct MB genetic subgroups, each one including unique survival, age, demographics, and genetic aberrations signature (Fig. 7, Table 1) (Northcott, Korshunov et al. 2011; Louis, Perry et al. 2016):

- MB WNT-activated
- MB SHH-activated and TP53-mutant
- MB non-WNT/non-SHH
- MB group 3
- MB group 4

Genetic profile	Histology	Prognosis
Medulloblastoma, WNT-activated	Classic	Low-risk tumour; classic morphology found in almost all WNT-activated tumours
	Large cell / anaplastic (very rare)	Tumour of uncertain clinicopathological significance
Medulloblastoma, SHH-activated, *TP53*-mutant	Classic	Uncommon high-risk tumour
	Large cell / anaplastic	High-risk tumour; prevalent in children aged 7–17 years
	Desmoplastic / nodular (very rare)	Tumour of uncertain clinicopathological significance
Medulloblastoma, SHH-activated, *TP53*-wildtype	Classic	Standard-risk tumour
	Large cell / anaplastic	Tumour of uncertain clinicopathological significance
	Desmoplastic / nodular	Low-risk tumour in infants; prevalent in infants and adults
	Extensive nodularity	Low-risk tumour of infancy
Medulloblastoma, non-WNT/non-SHH, group 3	Classic	Standard-risk tumour
	Large cell / anaplastic	High-risk tumour
Medulloblastoma, non-WNT/non-SHH, group 4	Classic	Standard-risk tumour; classic morphology found in almost all group 4 tumours
	Large cell / anaplastic (rare)	Tumour of uncertain clinicopathological significance

Table 1: Summary of the most common integrated medulloblastoma diagnoses, with clinical correlates (Louis, Perry et al. 2016).

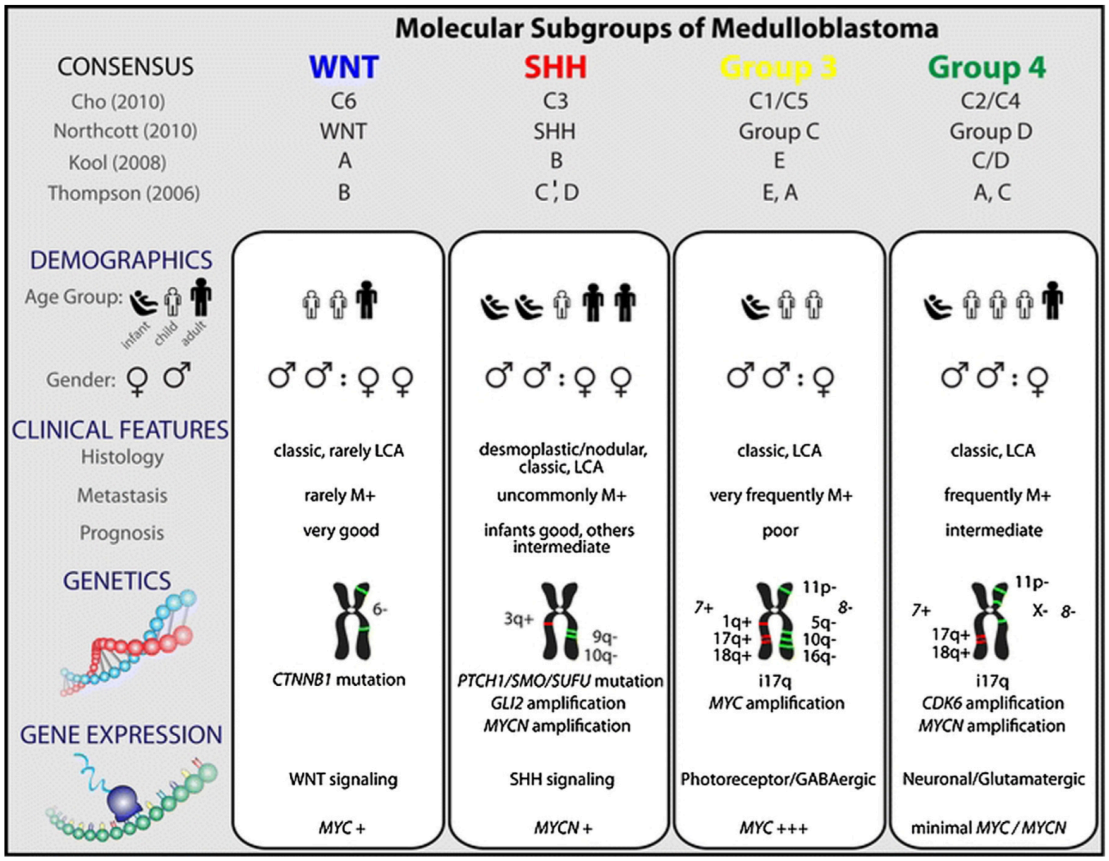

Fig. 7: MB molecular subgroups (Cho, Tsherniak et al. 2011)

WNT-activated MB

WNT-activated MB consists the least common group of MB, accounting of 11% of all cases (Kool, Korshunov et al. 2012). WNT MB patients are often older children (10-12 years) and unlike in the other sugroups, gender ratio presents a female preponderance (Taylor, Northcott et al. 2012). Histology is generally classic, and most tumors are non-metastatic. (Gajjar and Robinson 2014), (Salaroli, Ronchi et al. 2015).

In the WNT-activated subgroup, over 75% of tumors harbor a point mutation in the CTNNB1 gene (encoding beta-catenin) (Fig. 8), leading to hyper-activation of the WNT (Wingless) pathway by rendering beta-catenin resistant to degradation and causing nuclear accumulation of the protein and subsequent increased transcription of genes involved in cellular proliferation including cyclin D1 and c-Myc (Koch, Waha et al. 2001; Gilbertson 2004). Cytogenetically, WNT tumors are characterized by the deletion of one copy of chromosome 6 (monosomy 6) in the majority of patients (79% of the tumors) and tetraploidy (14% of the tumours) (Jones, Jager et al. 2012).

SHH-activated MB

Sonic Hedgehog (SHH) subgroup accounts for about 30% of all MBs and has a bimodal age distribution, occurring mostly in infants (< 3 years) and adults (>16 years), and less frequently in patients who are 3–16 years old (Gibson, Tong et al. 2010; Taylor, Northcott et al. 2012). The gender ratio is about 1:1, although there is a slight male predominance among infants (Gajjar and Robinson 2014). Histology is typically of the nodular/desmoplastic type with MBEN and confers excellent prognosis rates in infants and young children (Zeltzer, Boyett et al. 1999; Rutkowski, von Hoff et al. 2010; von Hoff, Hartmann et al. 2010; Lannering, Rutkowski et al. 2012). Patients in SHH group rarely have a disseminated tumor at diagnosis and generally have an intermediate prognosis, with a 5-year overall survival of approximately

75% when treated with standard therapy (Taylor, Northcott et al. 2012).

The main characteristic of SHH MBs is the activation of SHH signaling (Fig. 8), which is caused by mutations in PTCH1, SUFU, SMO, or other components of the SHH cascade.

Most common genetic aberrations in SHH subgroup include PTCH1 mutations (36–54% of the patients). However, SHH amplifications, mutations in SMO, SUFU, GLI2, MYC and MYCN genes also occur, with the latter comprising subgroups with a particularly poor prognosis (Eberhart, Kratz et al. 2002). Copy number aberrations include amplifications of protein phosphatase ID (PPM1D, chromosome 17q23.2), IGF1R, IRS2, PIK3C2G, PIK3C2B, and YAP-1 along with PTEN deletion on chromosome 10q23.31 and mutations in DDX3X (Northcott, Jones et al. 2012). Tetraploidy is present in about 29% of patients and it is associated with p53 mutations and chromothripsis (Jones, Jager et al. 2012).

Notably, TP53 mutations often occur among SHH MBs conferring a poor prognosis, thus SHH-activated MBs are considered to represent two distinct disease entities, depending on the TP53 genetic status. The two genetically distinct entities of SHH MB are ‘’MB SHH-activated and TP53-mutant’’ and ‘’MB SHH-activated and TP53-wildtype’’. The latter occurs mostly in adolescents and young children and has good prognosis rates. On the contrary, TP53-mutant SHH MBs occur in older children and have very poor prognosis (Ramaswamy, Remke et al. 2016).

non-WNT/non-SHH MB

This MB subtype represents the majority of MBs lacking either WNT or SHH pathway activation (non-WNT/non-SHH MBs). These tumors often lack recurrent mutations but show frequent chromosomal copy number alterations such as isochromosome 17q. They can be further subdivided with DNA methylation profiling or mRNA expression studies in “group 3” and “group 4” MBs. c-Myc amplification is frequently found in young children with group 3 tumors, and metastatic disease at diagnosis and is associated with poor outcome (Pietsch and Haberler 2016).

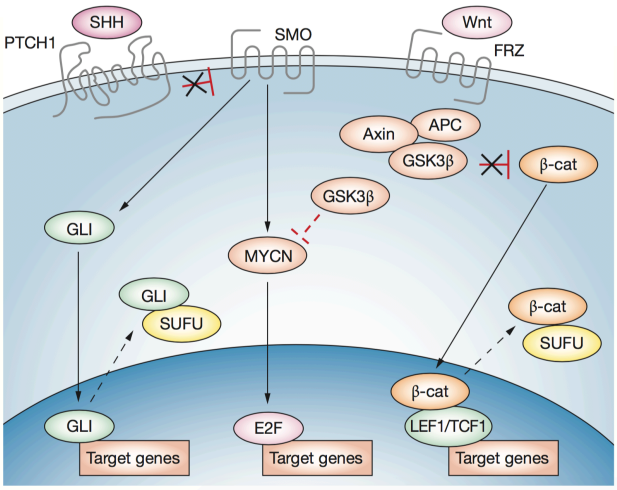

Figure 8: SHH and WNT signaling pathways implicated in the formation of MB. SHH signaling is initiated when SHH binds to its receptor, PTCH1, which releases SMO from inhibition, resulting in the activation of GLI and MYCN. Upon WNT binding to its receptor, FRZ, the APC complex is destabilized, allowing β-catenin to enter the nucleus and activate downstream transcription factors. Note the shared mechanisms of inhibition between the two pathways, SUFU and GSK3β. Abbreviations: APC: adenomatous polyposis coli, Axin: axis inhibitor protein, β-cat: β-catenin, FRZ: Frizzled, GLI: zinc finger protein GLI1, GSK3β: glycogen synthase kinase 3 β, LEF1/TCF1: lymphoid enhancer-binding factor 1/T-cell specific factor 1, MYCN: N-myc proto-oncogene protein, PTCH1: Patched 1, SMO: Smoothened, SUFU: Suppressor of Fused (Marino 2005).

Group 3 MB

Group 3 accounts for 25–28% of cases and is found almost exclusively in infants and children and

rarely in adolescents, presenting a slight male predisposition. Group 3 MB is the worst prognosis subgroup, with patients having a high incidence of metastatic disease at the time of diagnosis and less than 50% survival rates (Kool, Korshunov et al. 2012).

Most of these tumors harbor c-Myc amplifications which are responsible for aberrant MYC expression (Northcott, Jones et al. 2012). Genome instability is quite frequent and gains of 1q, 7, and 17q (i17q) are observed along with 10q, 11, 16q, and 17p deletions (Northcott, Jones et al. 2012). Tetraploidy is seen in 54% of group 3 tumours probably occuring as an early event in tumorigenesis (Jones, Jager et al. 2012). In a multivariate analysis, Shih and colleagues showed that the presence of i17q, MYC amplification, and presence of metastatic disease account for the poor outcome in this group (Shih, Northcott et al. 2014). There also appears to be upregulation of TGFβ signaling due to single copy number aberration of the genes involved in this signaling cascade (Northcott, Jones et al. 2012).

Group 4 MB

Group 4 is the highest incidence molecular MB subgroup, accounting for about 35% of all cases. Although this tumor type is found in all age groups, it is rarely found in infants, and presents a significantly male predominance (3:1) (Northcott, Jones et al. 2012).

Histologically, the vast majority of Group 4 MB are of classic histology, although cases of LC/A have also been observed (Ellison, Gajjar et al. 2011). Shih et al. have identified that loss of chromosome 11 and presence of i17q in a subset of patients with group 4 MB confer an excellent prognosis irrespective of metastatic disease (Shih, Northcott et al. 2014). However, the underlying biology of Group 4 MB is not well understood. Similar to group 3 tumors, tetraploidy occurs as an early change (40% of cases) (Jones, Jager et al. 2012). Isochromosome 17q occurs in 80% of cases, as does 17p deletion (Skowron, Ramaswamy et al. 2015). Additionally, chromatin-remodelling genes including KDM6A, ZYMYM3, and CHD7 are also mutated in this group. It has been reported that these mutations, along with overexpression of enhancer EZH2, keep neural stem cells in an undifferentiated state and might be sustaining tumorigenesis.

1.2.7.2 Epigenetics in MB

Lately, the unexpectedly high frequency and universality of alterations in the chromatin regulation machinery in MB and other malignancies has attracted significant research interest. Interestingly, alterations in genes involved in chromatin modifications (point mutations and DNA copy number aberrations) are a common characteristic across all MBs (Jones, Jager et al. 2012). Some of the identified epigenetic alterations include changes in chromatin-modifying genes, such as amplification of histone acetyltransferatses (KAT6A), lysine demethylases (JMJD2B, JMJD2C), deletions of histone methyltransferases (EHMT1, SMYD4), as well as H3K9 hypomethylation (Northcott, Nakahara et al. 2009).

Notably, there are observations supporting that epigenetic alterations occur in a subgroup-specific manner in MB. More specifically, truncating mutations of histone methyltransferases MLL2 and MLL3 have been identified in WNT MBs (Parsons, Li et al. 2011; Dubuc, Remke et al. 2013), whereas SHH MBs are strongly enriched for alterations in members of the nuclear receptor co-repressor (N-CoR) complex, which is associated with histone deacetylation and is thought to repress target genes by inducing chromatin condensation (Oberoi, Fairall et al. 2011).

Interestingly, TP53-mutated SHH MBs are often associated with chromothrypsis, an abnormality characterized by the loss of specialized chromatin structures at the telomeres, resulting in chromosome end-to-end fusions and subsequent mechanical shearing during mitosis (Tusell, Pampalona et al. 2010; Rausch, Jones et al. 2012).

On the other hand, group 3 and 4 MB subtypes share mutations of histone demethylases KDM6A and ZMYM (Lee, Wynder et al. 2005; Robinson, Parker et al. 2012). KDM6A acts in concert with the most

commonly mutated chromatin modifier in MB, MLL2, to increase H3K4me3 and remove H3K27me3 marks in order to target gene activation (Schuettengruber, Chourrout et al. 2007).

1.2.7.3 miRNAs in MB

miRNAs are short (19-25 nucleotides), evolutionary conserved RNA molecules that bind within the 3' untranslated region (UTR) of the messenger RNAs (mRNAs) and repress genes, either by blocking translation, or by directing sequence-specific degradation of target mRNAs (Bartel and Chen 2004; Chiang, Schoenfeld et al. 2010).

miRNAs have been proposed to be involved in cancer progression and altered miRNA expression profiles have been associated with outcome in several human cancers (Santarpia, Nicoloso et al. 2010). Since miRNA expression is deregulated in human malignancies, they may exert a dual function during the progression of the disease, acting either as oncogenes (by repression tumor-suppressor genes) or tumor suppressor genes (by repressing tumor-suppressor genes) (Zhang, Pan et al. 2007).

However, despite the importance of miRNAs in tumorigenesis, the first indications that miRNAs are implicated in MB pathogenesis emerged nine years ago, with miR124 being the first described miRNA to be involved in MB progression. miR-124, a modulator of cell cycle regulation (Pierson, Hostager et al. 2008), was found to be expressed at lower levels in MB samples compared to normal brain (Griffiths-Jones, Saini et al. 2008). Since then, there has been extensive research on the role of miRNAs in MB, and researchers could detect differential miRNA expression in the different MB molecular subgroups or histological subtypes, suggesting specific miRNAs as putative prognostic indicators.

More specifically, significant downregulation of 28 miRNAs has been demonstrated in SHH MB. Some of the identified miRNAs were miR-125b, miR-326, miR-214 and miR-324-5b and function as regulators of SMO and GLI1, which in turn are key activators of SHH cascade (Griffiths-Jones, Saini et al. 2008).

Furthermore, overexpression of miR-23b, miR-148a, miR-182, miR-193a, miR-224, and miR-365 was validated in WNT MBs, suggesting that the expression levels of these potential tumor/metastasis suppressive miRNAs could be important biomarkers for risk stratification in WNT MBs (Gokhale, Kunder et al. 2010).

Recently, the expression of 663 miRNAs was evaluated in MB primary samples, MB cell lines, neural stem cells as well as neural progenitor cells. The analysis revealed 33 differentially expressed (21 upregulated and 12 downregulated) miRNAs in MB primary samples compared to neural stem cells. Further integrative and functional analysis of predicted targets of the identified miRNAs revealed aberrations in MB signaling pathways related to neuronal migration, nervous system development and cell proliferation (Genovesi, Carter et al. 2011).

Cumulatively, the above findings indicate that miRNA profiling could be an important tool to support risk stratification and molecular classification of MBs and suggest that altering miRNA expression could serve as a novel therapeutic strategy for MB treatment.

1.2.7.4 Progenitors of the developing cerebellum

The developing cerebellum is composed of two major germinal zones: the ventricular zone (VZ) adjacent to the fourth ventricle, which gives rise to the majority of neurons and glia; and the external granule layer (EGL), around the outside of the cerebellum, which generates restricted populations of glutamatergic neurons (Fig. 9 & 10).

During embryogenesis, multipotent stem cells of the VZ undergo active proliferation and then differentiate to produce neuronal and glial progenitors. These cells radially migrate into the cerebellum and give rise to Purkinje, basket, stellate and Golgi neurons, as well as astrocytes and oligodendrocytes (Leto, Carletti et al. 2006). After birth, stem cells from the VZ migrate into the cerebellar white matter. Many of these cells become restricted to neuronal, oligodendroglial and astrocytic fates and ultimately give rise to mature neurons and glia (Milosevic and Goldman 2002).

During mid-gestation, a subset of VZ progenitor cells migrates laterally towards the upper rhombic lip (URL), where the expression of the transcription factor Atoh1 (Math1) is initiated under the influence of bone morphogenetic protein (BMP) signaling (Alder, Lee et al. 1999), leading thus to the restriction of Atoh1 to the neuronal lineage. The majority of URL progenitors then migrates around the outside of the cerebellum to form the EGL. Progenitor cells in the EGL (granule neuron precursors, or GNPs) proliferate extensively in response to SHH secreted by neighboring Purkinje cells. Ultimately (in response to signals that are poorly understood), GNPs exit the cell cycle, migrate inward to the internal granule layer (IGL), and differentiate into mature granule neurons, the most abundant type of neuron in the brain (Dahmane and Altaba 1999; Wallace 1999).

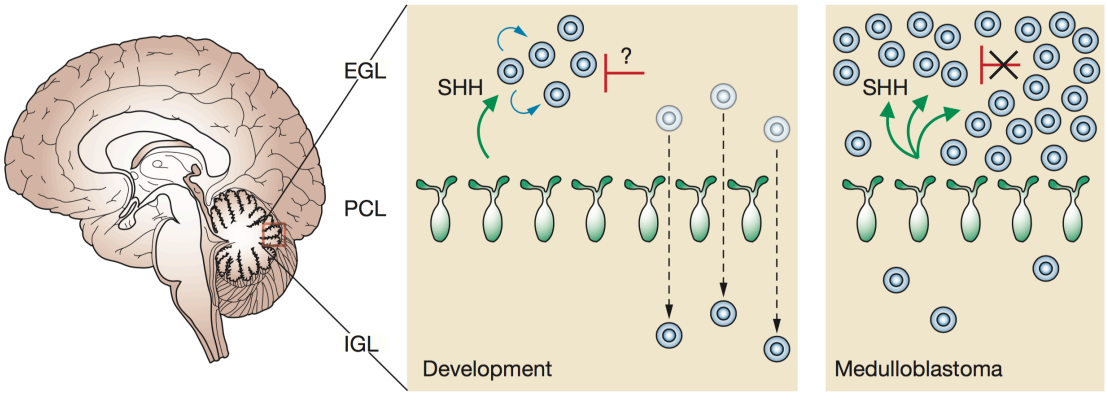

Figure 9: Granule-cell development and tumorigenesis of medulloblastoma. Under normal development, GCPs undergo massive proliferation in the external granule layer upon receiving the SHH signal from the Purkinje cell. GCPs then exit the cell cycle and begin to differentiate and migrate downward to form the IGL. Dysregulated granule cell development, including excessive signals for GCPs to proliferate or an absence of signals to cease dividing, can result in the formation of medulloblastoma. Abbreviations: EGL, external granule layer; GCP, granule cell precursor; IGL, internal granule layer; SHH, sonic hedgehog; PCL, Purkinje cell layer (Polkinghorn and Tarbell 2007).

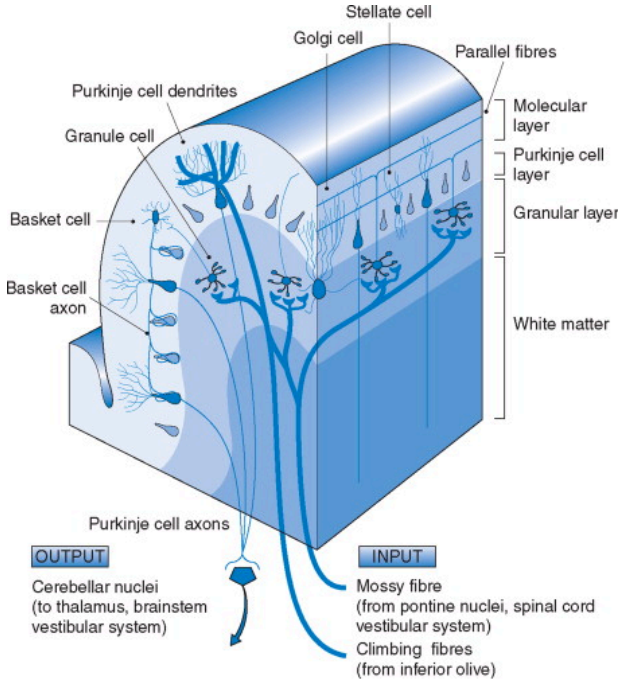

Figure 10: Three dimensional diagram of the principal circuitry within the cerebellar cortex. Input enters via the mossy and climbing fibers, while the only output is via the Purkinje cell axons (from JC Rothwell, in Physical Management in Neurological Rehabilitation (Second Edition), 2004).

1.2.7.5 Cells of origin for MB

SHH MB

The association between the SHH signaling pathway and MB was first recognized when Gorlin syndrome, a hereditary disease characterized by skin tumors, craniofacial abnormalities and an increased incidence of MB, was found to originate from mutations in Patched 1 (Ptch1) (Gorlin and Goltz 1960). Subsequently, mice with germline mutations in Ptch1 were reported to develop MB (Goodrich, Milenkovic et al. 1997), suggesting a causal role for SHH signaling in MB tumorigenesis. Later studies confirmed that GNP-specific deletion of Ptch1 or activation of Smoothened (Smo) results in MB formation, indicating that SHH-associated tumors arise from GNPs (Schuller, Heine et al. 2008).

WNT MB

Magnetic resonance imaging of patients with WNT MB demonstrated that this subtype of MB often encompasses the dorsal brainstem (Gibson, Tong et al. 2010). Moreover, WNT-associated tumors have a transcriptional profile resembling that of dorsal brainstem progenitors. These observations suggested that WNT-associated tumors might arise from the dorsal brainstem rather than the cerebellum. To test this hypothesis, Gibson and colleagues generated mice expressing a stabilized form of beta-catenin (Ctnnb1) (and lacking p53) in glial cells of the hindbrain. These animals exhibit defects in differentiation and migration of dorsal brainstem progenitors, with a subset prone to the development of tumors that resembled human WNT MB. Notably, activation of Ctnnb1 in GNPs does not cause increased proliferation or lead to tumor formation, suggesting that WNT-associated tumors do not arise from the granule lineage. Together these studies support the notion that WNT-associated MBs arise from dorsal brainstem progenitors (Wang and Wechsler-Reya 2014).

Group 3 MB

Although the cell of origin for these tumors remains unclear, much of current research has focused on the origins of group 3 MB characterized by c-Myc overexpression. Recent studies revealed that infection of cerebellar stem cells with c-Myc retroviruses and p53 mutants, led to the formation of tumors that histologically and molecularly resembled the c-Myc-driven group 3 MB (Pei, Moore et al. 2012). Similar results were acquired upon transplantation of cerebellar GNPs overexpressing c-Myc into mice cerebellum (Kawauchi, Robinson et al. 2012), suggesting that GNPs and stem cells give rise to group 3 MB.

Group 4 MB

Although group 4 is the most prevalent of the four MB subgroups, the origin of this specific subtype still remains unclear. Experiments performed in transgenic mice expressing MYCN in stem cells from the postnatal cerebellum, led to the development of tumors that exhibited classic or LC/A histology and resembled Group 4 MB at a molecular level (Swartling, Grimmer et al. 2010; Swartling, Savov et al. 2012), suggesting that the cell of origin for these tumors may be a primitive progenitor or stem cell.

1.2.8 MB targeted therapies

Genetically engineered models of MB

Genomic data have generated numerous hypotheses regarding the biology and appropriate treatments for each of the MB subgroups. One approach to test these hypotheses is by using genetically engineered mice (GEM) through which the function of particular genes in cancer development can be examined. Regarding MB, many existing GEM that have been created in order to study the function of putative MB oncogenes or tumor suppressors in stem cells or GNPs have yielded valuable information

about the origins and biology of the disease, and have provided valuable platforms for the evaluation of therapies.

Models representing the different subtypes of MB are described below.

Models of SHH MB

Among the first GEM models of MB was the heterozygous PTCH1 mice (Ptch1+/-mice). Approximately 15-20% of these mice develop cerebellar tumors that resemble human SHH MB (Goodrich, Milenkovic et al. 1997; Hahn, Wojnowski et al. 2000).

Notably, conditional *Ptch1*-knockout models of MB have also been developed, with mice developing MB with an incidence of 100%. In these animals, deletion of Ptch1 is either deleted specifically in GNPs (using Math-1-controlled Cre recombinase) or in stem cells (using GFAP-controlled Cre), representing a valuable tool for understanding the early stages of tumorigenesis in SHH MB (Yang, Ellis et al. 2008).

Models of MB have also been generated by targeting downstream elements of the SHH pathway. Of those, one of the most widely studied is the '*Smo-Smo*', which bears activated Smoothened (Smo) in neural progenitors or stem cells and confers high incidence, short latency and metastatic MBs, represents another important and valuable tool for preclinical studies. Additionally, animals lacking Sufu or overexpressing an activated form of Gli2 also develop MB (Tang, Gholamin et al. 2014).

An important use of SHH-associated mutations relies in defining genes that can cooperate with SHH signaling to promote MB formation. For example, crossing Ptch1+/- mice with Tp53-/- mice leads to a dramatic increase in tumor incidence, from 15% in Ptch1+/- mice to 100% in Ptch1+/-Tp53-/- mice. This penetrance has made the Ptch1+/-Tp53-/- model particularly useful for preclinical studies (Romer, Kimura et al. 2004).

Interestingly, analogous to Tp53, other oncogenes can cooperate with SHH signaling to promote MB tumorigenesis. More specifically, it has been reported that co-infection with viruses encoding C-myc, N-myc, insulin-like growth factor 2 (IGF-2), Akt, Bcl-2, or hepatocyte growth factor (HGF) leads to increased incidence of MB, suggesting that each of these genes is capable of cooperating with SHH pathway activation to promote tumor development (Rao, Pedone et al. 2003; Browd, Kenney et al. 2006; Binning, Niazi et al. 2008).

Models of WNT MB

Despite the generation of mouse models specifically expressing Ctnnb1 in the cerebellar VZ and in the lower rhombic lip, development of WNT MB was only achieved when the above mice were crossed with Tp53flx mice, aiming p53 deletion (which is often mutated in human WNT-associated tumours). Subsequent studies revealed that the addition of a phosphoinositide 3-kinase (PI3K) catalytic-α polypeptide mutant allele (Pik3caE545K) identified in WNT MB, the resulting mouse model presented highly penetrating WNT tumors, forming within 3 months. The above studies indicate that p53 as well as PI3K mutations consist hallmarks during WNT MB formation (Ellison, Onilude et al. 2005; Gibson, Tong et al. 2011; Bourdeaut, Miquel et al. 2014).

Models of other MB subtypes

In addition to models of SHH- and WNT-associated tumors, several models of non-SHH-associated tumors have recently been developed.

Studies have shown that deletion of *Rb* and *Tp53* in different cell types leads to tumor formation that displays different characteristics of SHH or WNT MBs, express stem cell markers such as Nestin, Sox2 and Sox9 and is associated with poor outcome (Sutter, Shakhova et al. 2010).

Notably, transgenic mice with targeted overexpressoin of n-Myc in brain astrocytes as well as cerebellum (Glt1-tTA::TRE-MYCN/Luc mouse model) presented aggressive MBs. The tumors were classified as group 3 MBs and showed large cell anaplastic histology, highlighting thus the agressiveness

of group 3 MB models and signifying the importance of n-Myc in MB. Furthermore the particular mouse model supports the important role of astrocytes in the development of the disease (Weiss, Grimmer et al. 2007).

Receptor tyrosine kinases (RTKs) and MB

Receptor tyrosine kinases (RTKs) serve as cell surface receptors for growth factors and cytokines. Upon ligand binding, receptor tyrosine kinases cluster and this leads to the activation of the protein's cytoplasmic domains. Activation can also be reinforced upon RTK dimerization in the case of certain RTKs. Trans-autophosphorylation of tyrosine residues within the receptor form binding sites for Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains. Phosphorylation of these proteins then results in the activation of downstream signaling pathways involved in diverse cellular responses, such as cell division, differentiation and motility. Members of the RTK family, specifically neurotrophin-3 receptor (TrkC), human epidermal growth factor receptor 2 (HER2/erbb-2), platelet derived growth factor receptor (PDGFR), insulin-like growth factor receptor (IGFR), nerve growth factor receptor (NGFR) as well as hepatocyte growth factor receptor (c-Met) have been associated with MB pathogenesis (Ng, Tong et al. 2003; Li, Lal et al. 2005).

1.3 HGF/c-Met signaling pathway

The HGF/c-Met pathway has been associated with normal development, organ regeneration and cancer. c-Met is a high affinity tyrosine kinase receptor for hepatocyte growth factor (HGF - also known as scatter factor). c-Met is generally expressed in epithelial cells and is activated by HGF produced in the surrounding mesenchymal cells or released into the circulation (Bladt, Riethmacher et al. 1995; Heymann, Koudrova et al. 1996). During embryogenesis, HGF/c-Met signaling is necessary for the development of tissues such as placenta, liver, kidney and neuronal tissue as well as for the directional migration of skeletal muscle cells (Birchmeier and Gherardi 1998). In adult tissues, the c-Met pathway has been implicated in regeneration and wound healing (Chmielowiec, Borowiak et al. 2007). Thus, the HGF/c-Met axis plays key role in cell proliferation, survival and migration, and when de-regulated, can give origin to a variety of cancers.

1.3.1 Structure of HGF and c-Met

HGF is synthesized as a single-chain inactive precursor and it is converted by serine proteases into an active form with two chains (α and β chain), linked by a disulfide bond. HGF consists of six domains: an amino-terminal hairpin loop domain (HL), four kringle domains (K1-K4) and a serine protease homology (SPH) domain which lacks enzymatic activity (Fig. 11) (Basilico, Arnesano et al. 2008).

c-Met, the HGF receptor, is extracellularly composed of three domains: the Sema domain, the PSI domain and four IPT domains. The intracellular region of c-Met consists of three portions: a juxtamembrane sequence that downregulates kinase activity upon phosphorylation of Ser975, a catalytic region that activates kinase activity following phosphorylation of Tyr1234 and Tyr1235, and a carboxy-terminal multifunctional docking site that contains two docking tyrosines (Tyr1349 and Tyr1356) essential for downstream signaling (Basilico, Arnesano et al. 2008).

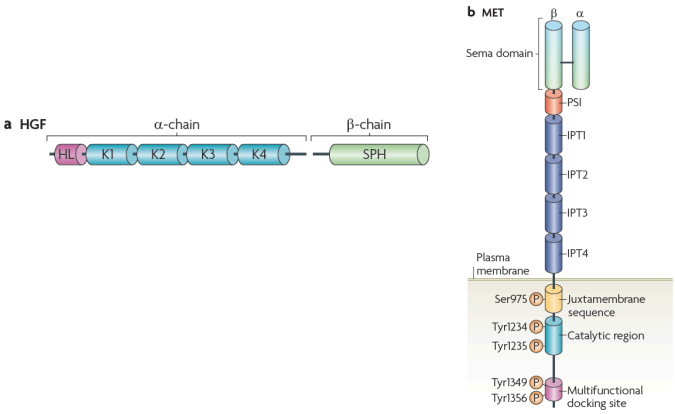

Figure 11: Structure of HGF growth factor and its transmembrane receptor c-Met: a) HGF consists of six domains: an amino-terminal hairpin loop (HL), four kringle domains (K1–K4; each defined by three conserved disulphide bonds) and a serine protease homology (SPH) domain that lacks proteolytic activity. **b)** The HGF receptor MET is a single-pass heterodimer comprising an entirely extracellular α-subunit that is linked by a disulphide bond to a transmembrane β-subunit, which contains the intracellular catalytic activity. The extracellular region of MET includes three functional domains: the Sema domain (which is also found in the semaphorins and plexins) spans the first 500 residues at the N terminus, encompassing the whole α-subunit and part of the β-subunit; the PSI domain (which is also present in the plexins, semaphorins and integrins, hence its name) covers approximately 50 residues and contains four conserved disulphide bonds; the residual 400 residues connecting the PSI domain to the transmembrane helix are organized into four IPT (immunoglobulin-like fold shared by plexins and transcriptional factors) domains. The intracellular segment is composed of three portions: a juxtamembrane sequence that downregulates kinase activity following phosphorylation of Ser975, a catalytic region that positively modulates kinase activity following trans-phosphorylation of Tyr1234 and Tyr1235, and a carboxy-terminal multifunctional docking site that contains two docking tyrosines (Tyr1349 and Tyr1356) that are involved in the recruitment of several transducers and adaptors.

1.3.2 c-Met signal transduction

Following HGF binding, the kinase activity of Met is switched on. This process starts with receptor dimerization and trans-phosphorylation of two tyrosine residues in the catalytic region (Tyr1234 and Tyr1235) and is followed by phosphorylation of two additional tyrosines in the carboxy-terminal tail (Tyr1349 and Tyr1356) (Fig. 12). These tyrosines create docking sites for a variety of adaptor proteins and direct kinase substrates such as the growth factor receptor-bound protein 2 (Grb2), Grb2-associated adaptor protein (Gab1), son of sevenless (SOS), SRC homology protein tyrosine phosphatase 3 (Shp2), phosphatidylinositol-3-kinase (PI3K) and signal transducer and activator of transcription 3 (STAT3). This leads to the activation of downstream signaling pathways that include the mitogen-activated protein kinase (MAPK), PI3K/AKT and STAT pathways (Ponzetto, Bardelli et al. 1994; Weidner, DiCesare et al. 1996; Lock, Royal et al. 2000).

The activation of the MAPK cascade will sequentially activate different protein kinases whose terminal effectors include extracellular signal-regulated kinases (Erk1 and Erk2), Jun amino-terminal kinases (JNK1, JNK2 and JNK3) and p38. These downstream elements activate cell cycle regulators leading to cell proliferation and promote alterations in cytoskeletal functions that control cell migration and invasion (Rodrigues, Park et al. 1997; Lamorte, Kamikura et al. 2000).

1.3.3 Regulation of c-Met signaling

It has been shown that the signaling network around the RTK c-Met is more complex than the known process of recruiting signaling effectors to the plasma membrane and subsequently stimulating intermediates in the cytosol. In fact, it has been found that relevant c-Met signals can also originate from endosomal compartments. It has been shown that HGF stimulates a rapid endocytosis of c-Met through clathrin coated vesicles in a dynamin dependent manner (Hammond, Urbe et al. 2001).

Upon HGF binding, c-Met is internalized by clathrin-mediated endocytosis and recruited into

peripheral early endosomes. This process is mediated by protein kinase Cε (PKCε) that promotes the transfer of active Erk to focal adhesions and, subsequently causes HGF-induced cell migration. From the peripheral endosomes, c-Met travels along the microtubule network to late perinuclear compartments in a process mediated by PKCα. This juxtanuclear accumulation of Met is a determinant step for activation and nuclear translocation of STAT3 (Kermorgant and Parker 2005).

1.3.4 HGF, c-Met and cancer

Dysregulated HGF/c-Met signaling has emerged as a key player in several human malignancies, particularly in tor cell invasion and metastasis. Human cell lines overexpressing either HGF and/or c-Met become tumorigenic and metastatic when implanted into nude mice (Rong, Segal et al. 1994). Moreover, transgenic mice expressing either the receptor or the ligand, develop metastatic tumors (Takayama, LaRochelle et al. 1997). On the contrary, downregulation of HGF or c-Met expression in human tumor xenografts decreases tumor growth (Abounader, Lal et al. 2001).

There are three biological mechanisms underlying the tumorigenicity of c-Met: a) the establishment of HGF/c-Met autocrine loops, b) the overexpression of HGF or c-Met, and c) the presence of activating mutations in the c-Met receptor (Ferracini, Direnzo et al. 1995).

The most frequent mechanism of c-Met deregulation found in human tumors is the overexpression of the receptor or its ligand. A large number of studies showed that HGF and c-Met are expressed in a wide variety of human tumors as well as in the metastasizing cells, conferring tumor aggressiveness and poor prognosis. These include malignancies of breast, colon, lung, pancreas and prostate, sarcomas, and glioblastomas (Direnzo, Olivero et al. 1995; Olivero, Rizzo et al. 1996; Tuck, Park et al. 1996; Koochekpour, Jeffers et al. 1997; Ramirez, Hsu et al. 2000; Tokunou, Niki et al. 2001; Tsao, Yang et al. 2001; Lengyel, Prechtel et al. 2005; Knowles, Stabile et al. 2009).

1.3.5 HGF/c-Met pathway in cerebellar development

The HGF/c-Met pathway also plays a critical role in cerebellar development. Comparing the regional expression of HGF and c-Met in both developing and adult rat brains, Achim and colleagues showed that HGF is expressed in the parietal cortex, striatum and cerebellar deep gray matter in the developing but not in the adult brain. Abundant expression of c-Met was also detected in the newborn cortex, thalamus and brainstem (Achim, Katyal et al. 1997). Leraci et al. described the expression of c-Met in the proliferating cells of the external granule layer of the cerebellum. In addition, transgenic mice with partial loss of c-Met function had a smaller cerebellum with abnormal foliation and balance impairment (Ieraci, Forni et al. 2002). Exploring the neuroprotective role of HGF, different groups showed that HGF treatment of primary cerebellar granule neurons prevents apoptotic cell death through activation of the PI3K/AKT pathway signaling (Zhang, Himi et al. 2000; Hossain, Russell et al. 2002).

1.3.6 HGF/c-Met pathway in MB formation

HGF/c-Met signaling has an important role in tumorigenesis and metastatic behavior in several human malignancies but its role in MB pathogenesis was only recently described.

Comparative genomic hybridization in a series of 14 human MB samples revealed amplification of the c-Met oncogene on chromosome 7q in 38.5% of cases (Tong, Hui et al. 2004). Further studies revealed that stimulation of MB cells with HGF increased activation of downstream effectors, evidenced by c-Met, MAPK and AKT phosphorylation. Up-regulation of the pathway was also able to induce cell proliferation, cell cycle progression, anchorage-independent growth and resistance to chemotherapy-induced apoptosis in MB cell lines. In vivo models overexpressing HGF also showed increased tumor

growth and invasion. Additionally, authors reported a positive correlation between the expression of HGF and c-Met in human MB samples and poor clinical outcome (Li, Lal et al. 2005).

Binning et al. showed that HGF and Shh cooperate to transform cerebellar neural progenitors in transgenic mice, leading to MB initiation and growth (Binning, Niazi et al. 2008). Last but not least, recent studies support the role of c-Met as a marker of SHH-driven MB. More specifically, based on the analysis of several large nonoverlapping cohorts of MB patients, active c-Met was correlated with increased tumor relapse and poor survival (Faria, Golbourn et al. 2015).

The above findings highlight the importance of the HGF/c-Met cascade in MB malignancies, further suggesting this pathway as a target for new promising therapies using small molecule inhibitors or antibodies.

1.3.7 Targeting HGF/c-Met pathway in cancer

Due to its pleiotropic role in cellular processes important in oncogenesis and cancer progression, HGF/c-Met cascade is considered to be a major target in anticancer therapy (Migliore and Giordano 2008; Trusolino, Bertotti et al. 2010). Several molecules targeting HGF/c-Met have been evaluated in early phase clinical trials. Most of them are small kinase inhibitors, while others include antagonists and monoclonal antibodies that target either the ligand or the receptor (Comoglio, Giordano et al. 2008; Eder, Woude et al. 2009). Based on the mechanisms of action, the HGF/c-Met agents can be classified into agents that either target HGF ligand, or those that target c-Met receptor.

HGF Regarding the agents targeting HGF, they are further categorized into either HGF activation inhibitors (preventing the cleavage of pro-HGF into the active form of HGF), or HGF inhibitors that block the direct binding of HGF to the c-Met receptor (Fig. 12 & 13). The activation of HGF from its inactive precursor pro-HGF is a critical step for HGF function (Gak, Taylor et al. 1992). This process is regulated by many proteases, including HGF activator (HGFA), matriptase, hepsin, and urokinase-type plasminogen activator (uPA), all of which convert inactive pro-HGF to active HGF. Two recently identified HGF activation inhibitors (HAIs), HAI-1 and HAI-2, have been shown to block HGF activation (Owen, Qiu et al. 2010; Fang and Lee 2014). HGF inhibitors bind and neutralize HGF, preventing thus HGF from binding to the c-Met and blocking the downstream signaling pathway. Some of the monoclonal antibodies that directly bind against HGF and have been studied in clinical trials include ficlatuzumab, HuL2G7, and rilotumumab (Tan, Park et al. 2011; Patnaik, Weiss et al. 2014). Of note, HuL2G7 (TAK-701) was one of the earliest mAbs developed which was shown to neutralize HGF (Jones, Cohen et al. 2010).

c-Met For agents targeting the c-Met receptor, they are classified into either c-Met antagonists that bind the receptor preventing thus the activation of downstream signaling components, or c-Met tyrosine kinase inhitors (TKIs) that target the ATP-binding site of the receptor and block receptor trans-autophosphorylation (Fig. 12 & 13). Although therapeutic strategies targeting c-MET should in principle be similarly efficacious to those targeting HGF, c-Met antagonists have been difficult to develop since many of them are bivalent (two-arm) antibodies that induce receptor crosslinking and subsequent downstream signal activation, thereby mimicking HGF ligand. Onartuzumab, the only monovalent MET antibody that has been tested in clinical studies, showed good tolerance when administered alone or in combination with bevacizumab in cancer patients (Salgia, Patel et al. 2014). Another approach towards the inhibiting of c-MET pathway is through c-MET kinase inhibitors, which target c-MET intracellularly. Interestingly, several small-molecule c-MET kinase inhibitors have entered clinical trials, including selective c-MET kinase inhibitors (e.g., tivantinib (ARQ 197) and nonselective c-MET kinase inhibitors (e.g., crizotinib (PF02341066), cabozantinib (XL 184), and foretinib). Notably, tivantinib (ARQ 197) has been the first non-ATP-competitive agent that selectively targets the c-MET RTK. In clinical trials, treatment with tivantinib has been generally well tolerated and has shown clinical activity in cancer patients (Feldman, Einhorn et al. 2013; Santoro, Simonelli et al. 2013; Kang, Muro et al. 2014; Pant, Saleh et al. 2014). INC 280 is another highly selective, small-molecule MET inhibitor which was well tolerated in a phase I study in

patients with advanced c-Met-dependent solid tumors such as non-small cell lung cancer, hepatocellular carcinoma etc (Bang, Su et al. 2014). Some of the c-MET nonselective, multikinase inhibitors include crizotinib that targets c-MET and ALK (Rodig and Shapiro 2010), cabozantinib targeting c-MET and VEGFR2 (Yakes, Chen et al. 2011), and foretinib that targets c-MET, VEGFR2 as well as PDGFR (Qian, Engst et al. 2009). Among the above compounds, cabozantinib strongly inhibited the self-renewal potential and in vivo growth of a c-MET-positive tumorigenic subpopulation of Giant cell tumor of bone (GCTB), suggesting that it could be an effective therapeutic option in patients with GCTB (Liu, Aleksandrowicz et al. 2014). Similar to cabozantinib, foretinib (GSK1363089) has also been tested in vitro and in vivo as to whether it could be used as a new therapeutic approach in SHH-driven MBs. Interestingly, foretinib suppressed MET activation, decreased tumor cell proliferation, and induce apoptosis in SHH medulloblastomas in vitro and in vivo. In established mouse transgenic or xenograft models of metastatic SHH, foretinib administration suppressed primary tumor growth, decreased the incidence of metastases, and increased host survival, suggesting that this compound should be clinically evaluated as an effective therapy for patients with SHH-driven MB (Faria, Golbourn et al. 2015).

Interestingly, current research regarding HGF/c-Met targeted cancer therapies seems to be inclined towards small molecule inhibitors compared to mAbs due to certain advantages of small molecules compared to mAbs. For instance, even though mAbs present high levels of specificity, their size restricts their ability to penetrate the cell membrane. Hence they are limited to targeting HGF or c-Met receptor only extracellularly. on the other hand, small molecules can cross the cell membrane and target intracellular targets. Furthermore, mAbs molecular weight varies significantly compared to small molecule inhibitors (150 kDa for mAbs vs. ~500 Da for small molecules). Thus mAbs present limited tissue distribution in comparison with small molecules, which can be extensively distributed into tissues due to their different chemical- and protein-binding properties. Thus, theoretically small molecules seem to have a therapeutic advantage for treatment of solid tumors possibly because they can be delivered into tumors relatively more easily than mAbs. Notably, to date the only US FDA-approved c-MET pathway-related agents, crizotinib and cabozantinib, are dual- or multi-tyrosine kinase inhibitors, indicating that blocking c-MET alone or in combination with other cancer-related pathways might be more beneficial for cancer treatment.

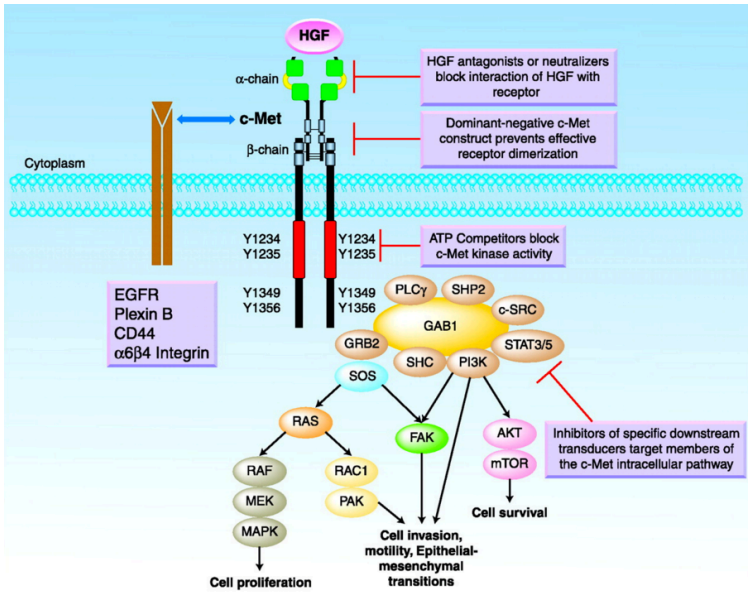

Figure 12: Major signaling pathways activated through c-Met, interactions between c-Met and other membrane receptors, and c-Met signaling inhibition strategies: HGF binding results in c-Met autophosphorylation of tyrosines Y1234 and Y1235 within the activation loop of the kinase domain and subsequent phosphorylation of tyrosines Y1349 and Y1356 near the -COOH terminus. Important adaptor proteins and direct kinase substrates activated downstream in the c-Met pathway include growth factor receptor-bound protein 2 (GRB2), Grb2-associated adaptor protein 1 (GAB1), phosphatidylinositol 3-kinase (PI3K), son of sevenless (SOS), rat sarcoma oncogene homolog (RAS), mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription 3/5 (STAT 3/5), SRC, SRC homology protein tyrosine phosphatase 2 (SHP2), SRC homology domain c-terminal adaptor homolog (SHC),

phospholipase c-γ (PLC), Ras-related C3 botulinum toxin substrate 1 (RAC1), p21-activated kinase (PAK), focal adhesion kinase (FAK), AKT, and mammalian target of rapamycin (mTOR). Crosstalk between c-Met and various membrane protein partners, including the epidermal growth factor receptor (EGFR), the plexin B family, α6β4 integrin, and CD44, results in additional signaling response modulation (Eder, Woude et al. 2009).

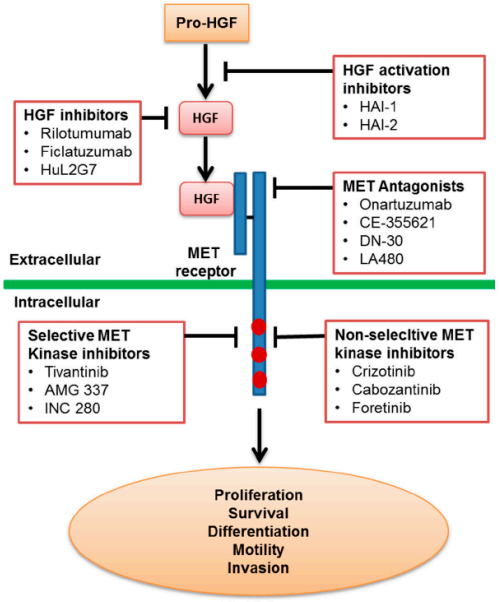

Figure 13: Representative examples of different mechanism classes of HGF and MET inhibitors (Zhang, Jain et al. 2015).

1.4 Radiation therapy

Conventional radiation therapy is usually applied with a beam composed of x-rays (photons) and leads to ionizations upon interaction of irradiation photons with biological matter. Subsequently, ionizing radiation (IR) produces high energy secondary particles (electrons), which can then trigger ionizations in the critical target molecules (e.g. DNA). The series of reactions that will follow take place in the cellular environment (due to its aquatic nature) and are known as ''Radiolysis of water'', leading to the production of free radicals. In the presence of oxygen, the free radicals will produce organic peroxides (reactive oxygen species – ROS), which are highly reactive. Therefore, IR may damage almost every cellular component, including proteins and lipids in the cell membrane (Stadtman 1993; Mishra 2002).

DNA damage, however, is the most relevant for cell killing (Hall 2006). DNA damage is classified into DNA dimers, oxidative base damage, intra- and inter-strand crosslinks, and single and double-strand breaks (DSBs). In order to repair the DNA damage, a cell has developed mechanisms for DNA damage sensing, that activate signal transduction pathways, which in turn orchestrate multiple cellular functions for successful damage repair (Wilson 2004).

Notably, among the different ways through which IR can lead to cell death, apoptosis is the one that can be induced with even low doses in some interphase cells. Apoptosis is an active process characterized by programmed cell death in which a defined cascade of events takes place. Various factors may modulate the apoptotic response to DNA damage, including the p53 status of the cell and levels and activity of the pro- and anti-apoptotic proteins. The ability to evade apoptosis is one of the hallmarks of cancer, however, there seems to be no direct correlation of apoptosis and radiosensitivity. A basic principle in the tumor radiobiology is the essential difference between cell death and loss of reproductive integrity of tumor cells, so-called clonogenicity, which is the most important read-out for the efficiency of curative radiation therapy (Kim, Emi et al. 2006). If cells do not undergo apoptotic death in interphase, they may become arrested in G1 and/or G2 phases of the cell cycle and then, as soon as the repair processes are accomplished, continue to proliferate. However, if the DNA damage was not sufficiently repaired during the arrest, the fate of the cell develops according to different scenarios that lead either to cell death or to the loss of clonogenicity:

I. Mitotic catastrophe: A result of aberrant mitosis, characterized by formation of nuclear envelopes around individual clusters of misaggregated chromosomes.
II. Senescence: A state when cells lose their ability to proliferate but keep on metabolizing.
III. Autophagy: Another type of programmed cell death which is characterized by the appearance of autophagic vacuoles in the cytoplasm.

Radiotherapy (RT) is one of the most prevalent cancer treatments. The goal of radiotherapy is to increase the total radiation dose to tumor tissue, while minimizing the toxicity in surrounding normal tissues. This goal can be best achieved by high accuracy conformal techniques and a sensitive fractionation schedule, where the total dose is applied in multiple (up to 35) small daily fractions. Fractionation helps to reduce dose limiting toxicities, by employing the major differences between cancer and normal cells: Most cancer cells are DNA damage repair (DDR)-impaired and proliferate more rapidly than most normal cells. It has been speculated that DDR inhibition might enhance the effectiveness of radiotherapy and DNA-damaging chemotherapies. Indeed, various DDR-inhibitory drugs are in preclinical and clinical development to test this premise (Dumont, Altmeyer et al. 2009).

1.4.1 Irradiation (IR): Friend or foe of antimetastatic therapy?

In spite of the continuous efforts to improve the accuracy of modern radiation therapy, normal tissue toxicity still has to be taken into consideration. Nowadays, acute side effects of RT are generally clinically manageable but late side effects (such as secondary tumors at the irradiated site) remain an important

issue especially for the treatment of childhood cancers including Medulloblastoma (Seehusen, Baird et al. 2010).

Another aspect, which is regularly reported in literature, is the potential pro-metastatic effect of IR. Although experimental evidence suggests that ionizing radiation can upregulate metastasis-related processes (Dumont, Altmeyer et al. 2009), however, there are no comprehensive studies of this problem and it is difficult to draw a firm conclusion from the existing reports because of the heterogeneity of patient cohorts (age, race and gender, tumor sites, staging), treatments applied (dose and fractionation of RT, target of RT, combination with surgery and chemotherapy), response to treatment (local failure or local control) and diagnosis of metastasis. However, the current consensus remains that under certain -still undetermined-circumstances, RT promotes metastasis.

Iradiation-induced malignant behavior is often represented by altered protein expression and activation profiles, therefore, various groups have tried to investigate possible links between radiation therapy and the activation of signaling cascades that lead to dissemination in different types of cancer.

The role of integrins

Integrin-mediated cell adhesion to ECM proteins is an essential mechanism of cell survival regulation in normal but also transformed cells (Cordes and Meineke 2003). Cordes et al. have examined whether β1 integrin-mediated adhesion confers resistance to radiation- or drug- induced genotoxic injury in A-172 glioma cells. They found that β1 integrin-mediated adhesion promotes cell survival post IR in an Akt-, p130Cas- and paxillin- dependent manner. Interestingly, IR-induced cell survival levels were reduced in cells expressing signaling-incompetent β1 integrin variants, or cells in which β1 integrin was downregulated. This suggested that the protein expression as well as the activity/phosphorylation signatures of adhesion proteins with low turnover, is highly critical and provides a basis for better understanding the mechanisms to cope with IR-genotoxic stress (Cordes, Seidler et al. 2006).

The role of c-Met

Due to the fact that c-Met is a known promoter of invasive growth, studies have focused on its function in IR-induced aggressive cell behavior. More specifically, De Bacco et al. have examined c-Met transcription and activation status after IR in human breast cancer and glioma cells. Notably, authors revealed that IR increased c-Met expression via activation of ATM and NF-kB. Furthermore, this IR-induced c-Met overexpression promoted ligand-independent c-Met phosphorylation and signal transduction, sensitizing thus the cells to HGF. Additionally, IR increased cell invasion in a c-Met dependent manner, since c-Met silencing by siRNA or pharmacological blockage using PHA665752 or JNJ-38877605 inhibitors counteracted radiation-induced increase of proliferation and invasiveness in vitro and in vivo and promoted apoptosis. The above results indicate that IR promotes the acquisition of a metastatic cellular phenotype and propose that combination of radiotherapy with c-Met inhibition can radiosensitize cancer cells. This finding is particularly relevant for clinical cases like glioblastoma and MB, where IR dosages are limited due to radiotoxicity (De Bacco, Luraghi et al. 2011).

IR and pancreatic cancer

Another type of cancer that presented IR-induced aggressive cellular behaviour is pancreatic cancer, therefore, numerous studies have been focused on the underlying molecular mechanisms (Qian, Mizumoto et al. 2002; Qian, Mizumoto et al. 2003). It has been shown that while coculture of pancreatic cancer cells with nonirradiated fibroblasts significantly increases the invasive ability of pancreatic cancer cells, the increased invasiveness is further accelerated when cancer cells are cocultured with irradiated fibroblasts. Interestingly, although irradiation did not affect fibroblast-induced HGF secretion, exposure of pancreatic cancer cells to supernatant from irradiated fibroblasts resulted in increased phosphorylation of

c-Met and MAPK activity, partially via increased expression of c-Met. In the same study, in vitro cell invasion assays revealed that scattering of pancreatic cancer cells was accelerated by the supernatant from irradiated fibroblasts, suggesting that invasive potential of certain pancreatic cancer cells is enhanced by soluble mediators released from irradiated fibroblasts, possibly through up-regulation of c-Met expression/phosphorylation and MAPK activity in pancreatic cancer cells (Ohuchida, Mizumoto et al. 2004). Furthermore, IR-enhanced invasiveness of pancreatic cancer cell lines has also been associated with increased expression and activity of MMP-2. Interestingly, MMP inhibitor CGS27023A, blocked the irradiation-induced invasive potential, suggesting that the concomitant use of MMP inhibitors during radiotherapy could potentially improve the efficacy of radiotherapy in pancreatic cancer (Qian, Mizumoto et al. 2002).

IR and breast cancer

Recent studies have revealed that irradiation-induced aggressive behavior of breast cancer cells is linked to tissue stiffness alterations. Asparuhova and colleagues have shown that tumors grown in pre-irradiated mammary glands were highly enriched in transcripts encoding collagens and other important components of the ECM, such as laminin, tenascins and MMPs. Notably, they identified the pro-metastatic megakaryoblastic leukemia-1 (Mkl1) protein (tenascin-C transcription regulator) to be a key mediator of a signaling cascade leading to the aggressive progression of mammary tumors locally relapsing after radiotherapy (Asparuhova, Secondini et al. 2015).

IR and endometrial carcinoma

EMT transition has been reported to be another consequence of irradiation in endometrial carcinoma. It has been shown that irradiation resulted in increased levels of invassiveness of endometrial carcinoma cells. Additionally, after irradiation cells exhibited changes resembling to EMT phenotype. These alterations included morphological changes as well as changes of the molecular level such as decrease in the expression of epithelial adhesion molecules (E-cadherin, β-catenin), or the increased expression of mesenchymal markers (fibronectin, vimentin), suggesting that radiation therapy can potentially promote EMT transition (Wild-Bode, Weller et al. 2001).

IR and Medulloblastoma

However, although radiation therapy is one of the most common MB therapeutic modality, little is known about potential irradiation-induced dissemination mechanisms in MB cells.

uPAR: Urokinase-type plasminogen activator receptor (uPAR) is another transmembrane receptor that is implicated in promoting signals related to cell survival, adhesion and migration (Smith and Marshall 2010). uPAR is known to form an active complex with integrins through the ligand uPA as well as vitronectin, playing thus a crucial role in activating integrin-mediated downstream signaling related to cell adhesion and migration (Yebra, Parry et al. 1996; Nguyen, Hussaini et al. 1998; Blasi 1999). Therefore, the contribution of the uPA/uPAR system in irradiation-induced MB cell aggressive behavior has also been examined. More specifically, it has been shown that irradiation enhances the invasiveness of MB cell lines and this correlates with increased uPAR proteolytic activity, increased expression levels of uPAR, uPA, FAK, N-Cadherin and α3, α5, β1 integrin subunits. Furthermore, uPAR co-localizes with β1 integrin at the migratory front of irradiated cells. Interestingly it has been reported that irradiation enhances uPAR association with β1 integrin and this leads to the activation of β1 integrin downstream signaling through FAK Tyr397 phosphorylation, which in turn leads to the activation of signals that enhance cell adhesion and invasion. Notably, FAK inhibition as well as uPAR downregulation abolished irradiation-induced invasiveness of MB cells, indicating that uPAR / β1 integrin / FAK axis control the increased irradiation-

induced cell invasion and migration of MB cells (Nalla, Asuthkar et al. 2010).

The role of radiation treatment in metastatic processes is a matter of great debate and seems to be strongly dependent on the tumor type and the experimental system that was used in the study. However, the pro-metastatic action of RT may explain why the better local control achieved by this treatment modality fails to be translated into longer survival, free of distant metastasis, and that the better radiotherapeutic strategies must aim at a better metastasis control (Dumont, Altmeyer et al. 2009).

1.5 Endocytosis

The routes that lead inward from the cell surface start with the process of endocytosis, by which cells take up plasma membrane components, fluid, solutes, macromolecules, and particle substances. Endocytosed cargo includes receptor-ligand complexes, a spectrum of nutrients and their carriers, extracellular matrix components, cell debris, bacteria, viruses, and even other cells. Through endocytosis, the cell regulates the composition of its plasma membrane in response to changing extracellular conditions (Conner and Schmid 2003; Doherty and McMahon 2009).

In endocytosis, the material to be ingested is progressively enclosed by a small portion of the plasma membrane, which first invaginates and is then pinched off to form an endocytic vesicle containing the ingested substance or particle. Endocytic vesicles form at the plasma membrane by multiple mechanisms that differ in both the molecular machinery used and how that machinery is regulated (Bonifacino and Traub 2003).

Once generated at the plasma membrane, most endocytic vesicles fuse with a common receiving compartment, the *early endosome*, where internalized cargo is sorted: Some cargo molecules are returned to the plasma membrane, either directly or via a *recycling endosome*, and others are designated for degradation by inclusion in a *late endosome*. Late endosomes form from a bulbous, vacuolar portion of early endosomes by a process called *endosome maturation* (Fig. 14). This conversion process changes the protein composition of the endosome membrane, patches of which invaginate and become incorporated within the organelles as intralumenal vesicles, while the endosome itself moves from the cell periphery to a location close to the nucleus. As an endosome matures, it ceases to recycle material to the plasma membrane and irreversibly directs its remaining contents towards degradation: Late endosomes fuse with one another and with lysosomes to form endolysosomes, which degrade their contents (Bonifacino and Traub 2003; Howes, Mayor et al. 2010; Kelly and Owen 2011).

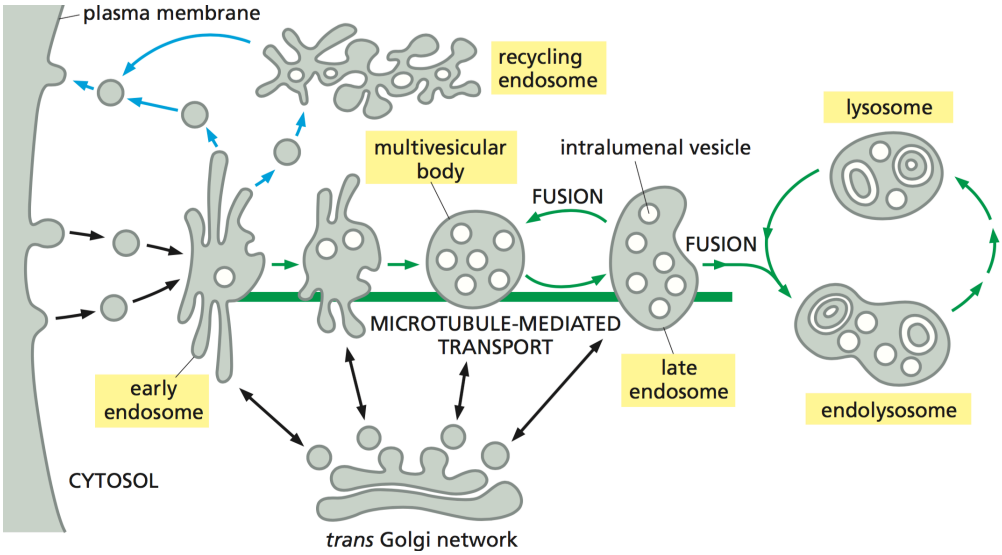

Figure 14: Endosome maturation: Endocytic processes initiating from the plasma membrane towards the lysosomes: Endocytic vesicles fuse near the cell periphery with an early endosome, which is the primary sorting station. Tubular portions of the early endosome bud off vesicles that recycle endocytosed cargo back to the plasma membrane—either directly, or indirectly via recycling endosomes. Membrane proteins destined for degradation are internalized in intralumenal vesicles. The developing late endosome, or multivesicular body, moves on microtubules to the cell interior. Fully matured late endosomes no longer send vesicles to the plasma membrane, and they fuse with one another and with endolysosomes and lysosomes to degrade their contents. Each stage of endosome maturation is connected via transport vesicles with the TGN (Trans Golgi Network), providing a continuous supply of newly synthesized lysosomal proteins (Alberts, Johnson et al. 2015).

1.5.1 Intracellular trafficking routes

Once cargo is internalized, the nascent vesicles are trafficked to various intracellular compartments for further sorting. In this way, the cell decides whether the cargo will be recycled back to the plasma membrane, or targeted for degradation (Maxfield and McGraw 2004). Independently of the entry route, the initial sorting compartment is often the early endosome. Upon sorting at the early endosome, cargo to be recycled can be trafficked back rapidly to the plasma membrane. Alternatively, cargo destined to be recycled may be trafficked through the perinuclear recycling endosome before being recycled to the plasma membrane. Instead of being recycled, some cargo protein and/or lipids are targeted for degradation. In this case, after sorting at the early endosome, these cargo molecules are trafficked to the late endosome or multivesicular body (MVB) and subsequently to the lysosome, where the cargo is ultimately degraded (Brown and Goldstein 1986).

Regarding the fate of the internalized cargo, it appears that it might depend on the cellular context. For example, ligand concentration may play a role in determining the fate of the receptor: low epidermal growth factor (EGF) concentrations favor recycling, while high ligand concentrations target EGFRs for degradation (Sigismund, Woelk et al. 2005; Sigismund, Confalonieri et al. 2012). Furthermore, over the past years it has been revealed that ubiquitination plays an important role in regulating the trafficking of growth factor receptors. While poly-ubiquitination through long chains of lysine 48-linked ubiquitin is well known to target proteins for degradation by the proteasome, modification of cargo proteins via mono-ubiquitination or short chain lysine 63-linked poly-ubiquitination, sometimes at multiple sites within the cargo, serves as a signaling module to target proteins for lysosomal degradation (Grant and Donaldson 2009; Clague, Liu et al. 2012)

1.5.2 Rab proteins

To ensure an orderly flow of vesicle traffic, transport vesicles need to display surface markers that identify them according to their origin and type of cargo. Furthermore, target membranes need to present complementary receptors that recognize the appropriate markers. This crucial process occurs in two steps: First, Rab proteins and Rab receptors direct the vesicle to specific spots on the correct target membrane. Second, SNARE proteins and SNARE regulators mediate the fusion of the lipid bilayers (Martens and McMahon 2008; Pfeffer 2013).

Rab proteins play a central part in the specificity of vesicle transport. With over 60 known members, the Rab family is the largest of the monomeric GTPase subfamilies. Each Rab protein is associated with one or more membrane-enclosed organelles of the secretory or endocytic pathways, and each of these organelles has at least one Rab protein on its cytosolic surface (Table 2). Their highly selective distribution on these membrane systems makes Rab proteins ideal molecular markers for distinguishing membrane type and trafficking vesicles between them (Pfeffer 2013; Pfeffer 2017) (Grosshans, Ortiz et al. 2006).

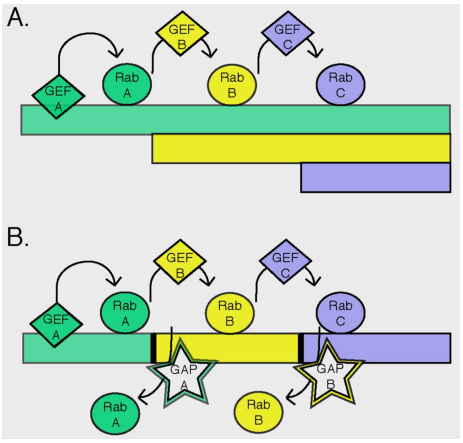

Figure 15: Rab cascade model for the establishment and maintenance of the polarity of the secretory and endocytic pathways. (A) In the first scenario, a Rab GEF specific for Rab A generates active Rab A. That Rab recruits a second GEF, which activates Rab B. Similarly, Rab B recruits a GEF to activate Rab C. In this model, a membrane could have Rabs A–C intermingled or at least on a single compartment. **(B)** Here GAP proteins are included to remove a previous-acting Rab from a specific membrane domain. The presence of the GAP will sharpen the boundaries between individual Rab domains. (Pfeffer 2017).

Protein	*Organelle*
Rab1	ER and Golgi complex (Batoko, Zheng et al. 2000)
Rab2	Cis Golgi network (Short, Preisinger et al. 2001)
Rab3A	Synaptic vesicles, secretory vesicles (Vadlamudi, Wang et al. 2000)
Rab4/Rab11	Recycling endosomes (Li, Li et al. 2008)
Rab5	Early endosomes, plasma membrane, clathrin-coated vesicles (Jovic, Sharma et al. 2010)
Rab6	Medial and *trans* Golgi (Storrie, Micaroni et al. 2012)
Rab7	Late endosomes (Vanlandingham and Ceresa 2009)
Rab8	Cilia (Lu, Insinna et al. 2015)
Rab9	Late endosomes, Trans Golgi (Barbero, Bittova et al. 2002)

Table 2: Subcellular Locations of certain Rab proteins

1.5.3 Rab protein effectors

Rab proteins can function on transport vesicles and on target membranes, or both. Rab proteins cycle between a membrane and the cytosol and regulate the reversible assembly of protein complexes on the membrane. In their GDP-bound state, they are inactive and bound to another protein (Rab-GDP dissociation inhibitor, or GDI) that keeps them soluble in the cytosol (Fig. 15). In their GTP-bound state, they are active and tightly associated with the membrane of an organelle or a transport vesicle. Membrane-bound Rab-GEFs activate Rab proteins on both transport vesicle and target membranes. Once in the GTP- and membrane-bound state, Rab proteins bind to other proteins, called Rab effectors, which are the downstream mediators of vesicle transport, membrane tethering, and membrane fusion. The rate of GTP hydrolysis sets the concentration of active Rab and, consequently, the concentration of its effectors on the membrane (Zerial and McBride 2001; Grosshans, Ortiz et al. 2006).

In contrast to the highly conserved structure of Rab proteins, the structures and functions of Rab effectors vary greatly, and the same Rab proteins can often bind to many different effectors. Some Rab effectors are motor proteins that propel vesicles along actin filaments or microtubules to their target membrane. Others are tethering proteins, some of which have long, threadlike domains that serve as “fishing lines” that can extend to link two membranes more than 200 nm apart. Other tethering proteins are large protein complexes that link two membranes that are closer together and interact with a wide variety of other proteins that facilitate the membrane fusion step. Coupling uncoating to vesicle delivery helps to ensure directionality of the transport process and fusion with the proper membrane. Rab effectors can also interact with SNAREs to couple membrane tethering to fusion (Jahn and Scheller 2006; Harrison and Kirchhausen 2010; Jin, Pahuja et al. 2012).

1.5.4 Rab implication in the generation of different membrane domains

The assembly of Rab proteins and their effectors on a membrane is cooperative and results in the formation of large, specialized membrane patches. Rab5, for example, assembles on endosomes and mediates the capture of endocytic vesicles arriving from the plasma membrane. The experimental

depletion of Rab5 causes disappearance of the entire endosomal and lysosomal membrane system, highlighting the crucial role of Rab proteins in organelle biogenesis and maintenance (Zeigerer, Gilleron et al. 2012). The assembly of a Rab5 domain on endosomal membranes begins when a Rab5-GDP/GDI complex encounters a Rab-GEF. GDI is released and Rab5-GDP is converted to Rab5-GTP. Active Rab5-GTP is anchored to the membrane and recruits more Rab5-GEF to the endosome, thereby stimulating the recruitment of more Rab5 to the same site (Zerial and McBride 2001). In addition, active Rab5 activates a PI3-kinase, which locally converts PI to PI(3)P, which in turn binds some of the Rab effectors including tethering proteins and stabilizes their local membrane attachment (Jean and Kiger 2012). This type of positive feedback greatly amplifies the assembly process and helps to establish functionally distinct membrane domains within a continuous membrane.

The endosomal membrane provides a striking example of how different Rab proteins and their effectors help to create multiple specialized membrane domains, each fulfilling a particular set of functions. Thus, while for instance the Rab5 membrane domain receives incoming endocytic vesicles from the plasma membrane, distinct Rab11 and Rab4 domains in the same membrane organize the budding of recycling vesicles that return proteins from the endosome to the plasma membrane.

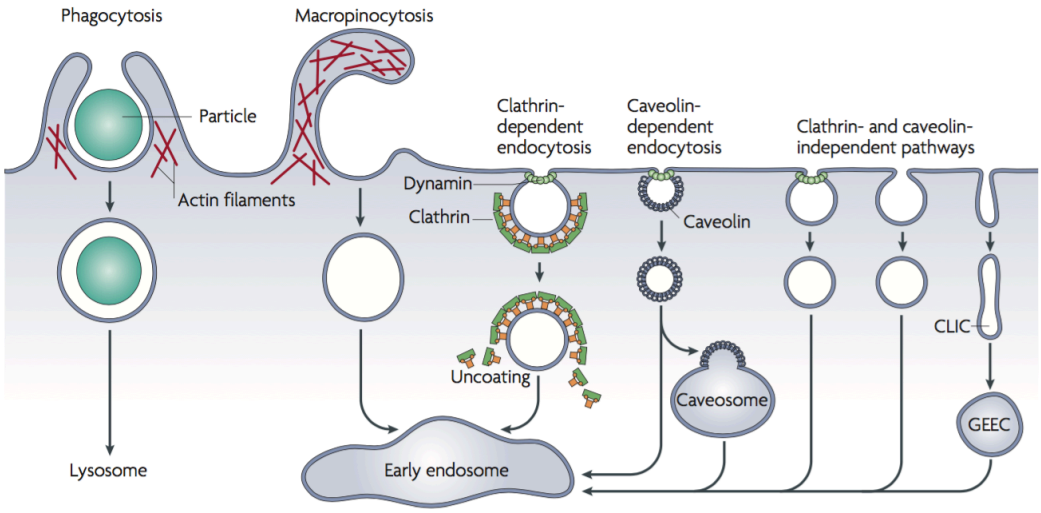

Figure 16: Pathways of endocytosis: Large particles can be taken up by phagocytosis, whereas fluid uptake occurs by macropinocytosis. Both processes appear to be triggered by and are dependent on actin-mediated remodelling of the plasma membrane at a large scale. Compared with the other endocytic pathways, the size of the vesicles formed by phagocytosis and macropinocytosis is much larger. Numerous cargoes can be endocytosed by mechanisms that are independent of the coat protein clathrin and the fission GTPase, dynamin (Mayor and Pagano 2007).

1.5.5 Mechanisms of endocytosis

The two general mechanisms by which extracellular material enters the cell are clathrin-mediated endocytosis (CME) and the clathrin-independent endocytic process, CIE (Fig. 16) (Cooper and Shaul 2006; Doherty and McMahon 2009). CME is well known for its role in the internalization of growth factor receptors and is dependent on clathrin coat formation as well as the recruitment of accessory proteins that recognize specific cytoplasmic sorting sequences. In contrast, CIE appears to be responsible for mediating the internalization of a variety of substrates, is less well defined and most importantly, lacks the requirement for clathrin or specific internalization signals. CIE includes phagocytosis, micropinocytosis, caveolae-dependent endocytosis and clathrin/caveolae-independent endocytosis (CCI) (Maxfield and McGraw 2004; Schmid and McMahon 2007; Doherty and McMahon 2009; McMahon and Boucrot 2011; Reider and Wendland 2011; Schroeder and McNiven 2014).

1.5.5.1 Clathrin-Mediated Endocytosis (CME)

CME, often referred to as receptor-mediated endocytosis (RME), is characterized by the formation of rounded shallow clathrin-coated pits (CCPs) (Fig. 17) at the plasma membrane. Clathrin-coated pits consist of clathrin and AP-2 proteins, with Arf6 GTPase being a potential regulator of this process (Krauss, Kinuta et al. 2003). AP-2 comprises an indispensable component of clathrin-coated pits and is involved in cargo selection, in vesicle assembly (Robinson and Bonifacino 2001), as well as in the recruitment of clathrin and accessory proteins.

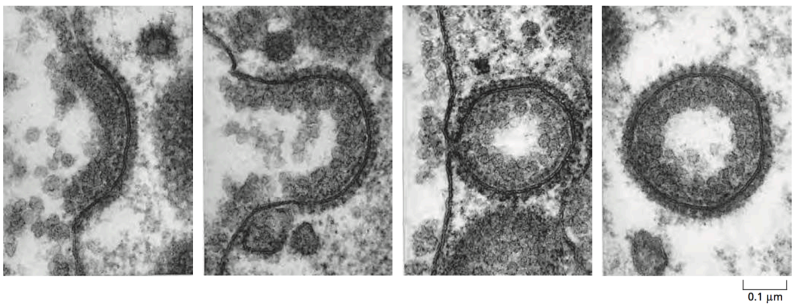

Figure 17: Clathrin-Mediated Endocytosis (CME): Illustration of events occurring during CME of lipoprotein particles in a hen oocyte during yolk formation. The formation of a clathrin-coated pit results in the generation of a clathrin-coated vesicle. The lipoprotein particles bound to the membrane receptors appear as a dense, fuzzy layer on the extracellular surface of the plasma membrane—which will become the inside surface of the coated pit and vesicle (McMahon and Gallop 2005).

CME initiation is defined by the formation of clathrin-coated pits. Subsequently, adaptor proteins bind to vesicle-coating proteins and to the cytoplasmic domains of membrane proteins, thereby linking specific cargoes to sites of coat assembly.

After the assembly, clathrin-coated vesicle needs to be pinched off the membrane and released into the cytosol, which requires the involvement of various proteins with different functions. At first, accessor proteins such as epsins, endophilin and amphiphysin (Wigge and McMahon 1998; Farsad, Ringstad et al. 2001; Ford, Mills et al. 2002) induce membrane bending, which is required for the initiation of vesicle formation. Dynamin 2 (Dyn2) then mediates the scission of the vesicle from a donor membrane with subsequent release in the cytoplasm, comprising thus an indispensable molecule for proper CME (Hinshaw 2000; Praefcke and McMahon 2004).

Another component that participates in the release of coated vesicles from the plasma membrane is the cortical actin cytoskeleton. Actin motors for example, such as the minus-end motor Myosin VI and the plus-end motor Myosin 1E have been shown to participate in CME via the interaction with the adaptor Dab2 and PdtIns(4,5)P2 or Dyn2 and synaptojanin 1, respectively (Krendel, Osterweil et al. 2007; Spudich, Chibalina et al. 2007). Other actin links to the endocytic machinery include actin-binding proteins such as profilin, synapsin, syndapin, and cortactin, all of which, surprisingly, are known binding partners of dynamin as well (Molloy, Thomas et al. 1994; Johnson, Subtil et al. 1998).

Finally, clathrin coat is removed by energy-consuming mechanism, which eventually leads to the fusion with the early endosomes. Endosomal sorting regulates whether the contents are recycled back to the membrane, or continue towards the endosomal maturation process to ultimately end up with lysosomal degradation.

Vesicle formation and trafficking also involves a cooperative and dynamic interaction between lipids and proteins. The specific lipid composition of membranes affects membrane fluidity, receptor clustering, and signaling of the coating and accessory proteins. To facilitate this lipid-protein interaction, a number CME-associated proteins contain lipid-binding domains. In particular, PIP2, owing to its phosphoinositide-containing membrane domains, is necessary for targeting and localizing the α- and µ2- adaptin subunits of AP-2 to the plasma membrane. Additionally, the interaction of Dyn2 with PIP2, is important during tubulation and vesicle scission processes (Mim and Unger 2012; Chappie and Dyda 2013; Meinecke, Boucrot et al. 2013).

Notably, alternate endosomal fusion is a process that requires SNARE and Rab proteins (van Dam, ten Broeke et al. 2002), as well as protein assemblies that are similar to those involved in clathrin-coated-

vesicle formation. For instance, the SNARE protein cellubrevin (VAMP3) is required for the fusion of transferrin-receptor-containing vesicles with the plasma membrane (Galli, Chilcote et al. 1994), while there is evidence that dominant-negative Dyn2 mutants inhibit transferrin-receptor cycling, highlighting thus the requirement for Dyn2 towards the formation of recycling vesicles (van Dam and Stoorvogel 2002; van Dam, ten Broeke et al. 2002).

1.5.5.2 Clathrin-independent endocytosis (CIE)

Various types of CIE have been described, while the factors involved depend on the cargo and cell type examined. Some of the proteins implicated in CIE include important components of the plasma membrane, components of the immune response (e.g. MHCI and II), transporters (e.g. calcium and potassium channels, glucose, and amino acid transporters), growth factor receptors (e.g. β-adrenergic receptor and c-Met) or cell adhesion components (e.g. ICAM1, E-Cadherin and integrins) (Sandvig, Pust et al. 2011; Maldonado-Baez, Williamson et al. 2013).

Depending on the cargo protein, CIE processes are regulated by Rho GTPases such as Cdc42 and Rac1, RhoA (IL-2Rβ) or Arf6, and may also be either dependent or independent of dynamin-2 function (Howes, Mayor et al. 2010). Interestingly, CIE cargo proteins merge with CME cargoes in Rab5 and EEA1 positive early endosomes. Moreover, recycling of CIE cargo is controlled by other members of the Rab GTPases such as Rab11 and Rab22a (Naslavsky, Weigert et al. 2003; Weigert and Donaldson 2004; Weigert, Yeung et al. 2004; Naslavsky, Rahajeng et al. 2006). CIE pathways include phagocytosis, caveolae-mediated uptake, macropinocytosis and constitutive non-clathrin uptake.

Phagocytosis

Phagocytosis is a cellular process that processes solid particles of usually at least 0.5 mm in diameter and is generally restricted to macrophages and other phagocytes that specialize in uptake and digestion of large particles (Mellman 2000).

Phagocytosis is initiated when cells use a specific receptor protein to recognize and bind particles. Receptor choice is defined by recognition patterns presented on phagocytic particles (defined as particles that trigger their engulfment by phagocytic cells, e.g. pathogens), and by availability of appropriate levels of opsonins - soluble multidomain proteins that bridge particle and phagocytic cell by binding to both particle recognition patterns and phagocyte receptors. Depending on the nature of the recognition/binding proteins, the engulfment of surface-bound particles is followed by the recruitment of additional cell surface as well as cytosolic proteins and leads to particle internalization and formation of an intracellular membrane-enclosed organelle, a phagosome. Two principle types of phagocytosis exist and are described below.

Anti-pathogen phagocytosis

Anti-pathogen phagocytosis generally occurs in the immune cells and serves both for the removal of microbes and the recruitment of numerous immune cells through complex cytokine and chemokine secretion, to establish an effective anti-inflammatory defense. Anti-pathogen phagocytosis is mostly performed by phagocytes, such as immature dendritic cells (DCs) and macrophages as well as by tissue-resident immune cells (Reddick and Alto 2014).

Clearance phagocytosis or efferocytosis

This type of phagocytosis is neither anti-inflammatory nor immunogenic (Rothlin, Ghosh et al. 2007) and is also performed by macrophages and DCs. Following apoptosis, dead cells need to be taken up into the surrounding tissues through a process called efferocytosis (deCathelineau and Henson 2003). The main feature of an apoptotic cell is the cell surface presentation of a variety of intracellular molecules such as calreticulin, phosphatidylserine, annexin A1, oxidized LDL and altered glycans (Henson 2005). These molecules are recognized by macrophage cell surface receptors such as thrombospondin 1, Growth arrest-specific (6GAS6) and lactadherin (MFGE8), which subsequently bind to other macrophage receptors such as CD36 and αvβ3 integrin. Defects in apoptotic cell clearance are usually linked with impaired phagocytosis of macrophages and often lead to autoimmune disorders (Kruse, Janko et al. 2010; Roszer, Menendez-Gutierrez et al. 2011)

Although the above types of phagocytosis are composed of different particles and have different functions, they share many molecular characteristics. Both forms of phagocytosis share the recruitment of F-actin structures, that is called phagocytic cup beneath the cell surface-bound particles upon the initiation of the process. Subsequently, cell surface shape changes locally (usually opsonins) and wraps around the phagocytic target. The closure of the phagocytic cup requires F-actin disassembly and leads to particle internalization and phagosome formation. Then, alternate series of fusion and trafficking events result in the maturation of phagosomes first in phagolysosomes and then to lysosomes (Swanson 2008).

Caveolae-mediated uptake

Central components of clathrin-independent uptake are small, flask-shaped invaginations of the plasma membrane named caveolae. Caveolae are involved in trans-endothelial transport, organization of plasma membrane domains, and may also serve as platforms for different signaling cascade networks. They are especially abundant in endothelial cells, where they are implicated in transcytosis and endocytosis of blood components. (Frank, Woodman et al. 2003; Parton and Simons 2007; Boscher and Nabi 2012).

Biochemically, caveolae are characterized by their association with a family of cholesterol-binding proteins called caveolins. Interestingly, expression of caveolin in cells that do not normally have caveolae is apparently sufficient to generate these structures (Brett, Legendre-Guillemin et al. 2006).

In addition to their role in endocytosis of specific molecules, caveolae have been found to co-localize with several membrane receptors, signaling molecules and membrane transporters, suggesting that they might also act as sites of signaling activity within cells (Ceresa and Schmid 2000).

The extent and mechanism by which caveolae mediate uptake of molecules within cells has been controversial. It is known that Caveolae-mediated uptake relies on the ability of caveolins to oligomerize and this leads to the formation of caveolin-rich microdomains, which along with increased levels of cholesterol lead to expansion of the caveolar invagination and to formation of endocytic vesicle (Fig. 18). Fission of the vesicle from the plasma membrane is then mediated by GTPase dynamin II which is localized at the neck of the budding vesicle. The released caveolar vesicle can fuse with early endosome or caveosome. The caveosome is an endosomal compartment with neutral pH which does not have early endosomal markers, however, contains molecules internalized by the caveolar endocytosis. This type of endocytosis is used for example for transcytosis of albumin in endothelial cells or for internalization of the insulin receptor in primary adipocytes (Parton and Simons 2007; Lajoie and Nabi 2010).

Potocytosis, involving direct diffusion of small molecules into the cytoplasm after association with caveolae, has been proposed as an alternative way caveolae mediate uptake of molecules without membrane internalization. The finding that caveolae contain one key element of the machinery involved in vesicle budding, the GTPase dynamin, suggests that they also participate in membrane internalization.

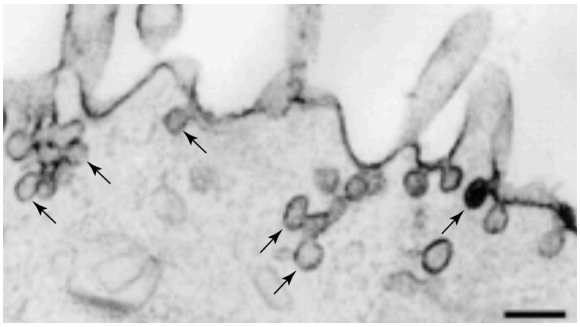

Figure 18: Caveolae. Caveolae accumulating in a cell micro-injected with antibodies against dynamin 2 (Calzolari, Raggi et al. 2006).

Insight into the mechanism of caveolar internalization of cell-surface molecules has been obtained by visualizing the trafficking of cargo that internalizes through caveolae. The best characterized caveolae-internalized cargo is SV40 virus, which was found to associate with the plasma membrane in a two-step process, first with binding to mobile MHC I antigens and then becoming trapped in stationary caveolae (Babst 2005).

Macropinocytosis

Macropinocytosis refers to the formation of large, primary endocytic vesicles, generated primarily at ruffling membrane domains. Macropinosomes are dynamic structures, frequently moving inwards towards the centre of the cell, and persist for approximately 5–20 min (Gong, Weide et al. 2007). Macropinocytosis is a signal-dependent process that usually occurs in response to growth factor stimulation, such as macrophage colony-stimulating factor-1 (CSF-1), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Haigler, Mckanna et al. 1979; Racoosin and Swanson 1989). The main characteristic of macropinocytosis is the involvement of actin-mediated membrane ruffling of the plasma membrane. Generally, most of the lamellipodia formed retract back to the cell. However, a subset of lamellipodia may fold back onto themselves and fuse with the basal membrane creating large, shaped vesicles named macropinosomes (Fig. 19).

Macropinosomes are distinct from other forms of endocytic vesicles in the sense that they have no apparent coat structures and that they are generally larger than other endocytosis-related structures (such as clathrin-coated vesicles) (Hewlett, Prescott et al. 1994; Swanson and Watts 1995). Owing to their large size, macropinosomes, provide the cells with cells with the capability of non-selectively internalizing large quantities of solute and membrane.

The primary regulator of membrane recycling between the plasma membrane and endosomal compartments during macropinocytosis is considered the GTPase Arf6 (Radhakrishna and Donaldson 1997; Franco, Peters et al. 1999). Notably, studies have demonstrated that overexpression of either mutant Arf6 locked in its GTP-bound form, or the EFA6 GDP–GTP exchange factor for Arf6, results in both ruffling and the accumulation of macropinosomes. Arf6 seems to orchestrate macropinocytic processes by regulating the turnover and activation of different effectors. More specifically, Arf6 activates phosphatidylinositol 4-phosphate 5-kinase (PtdIns(4,5)P2) at the plasma membrane (Honda, Nogami et al. 1999), suggesting that PtdIns(4,5)P2 turnover is likely to be one important means by which the dynamics of macropinosomes are regulated. Consistent with this, constitutive macropinocytosis in oncogene-transformed fibroblasts requires constant PtdIns(4,5)P2 turnover. Furthermore, the spectrin–ankyrin-based cytoskeleton provides an additional class of potential ARF6 effectors, since spectrin-like ligands bind spectrin SH3-domain-binding protein 1 (Hssh3bp1) to the macropinosomes, suggesting that ARF6 activation affects both membrane trafficking and cortical actin dynamics (Xu, Ziemnicka et al. 2000). Once formed, macropinosomes undergo a maturation process which is briefly depicted in Fig. 19. However, this maturation process occurs in a different way depending on the cell type.

In bone marrow-derived macrophages, macropinosomes are formed in response to treatment with CSF-1 and within minutes they start acquiring Rab7 late endosomes marker, before their fusion with lysosomes and eventual degradation (Racoosin and Swanson 1989). However, Unlike bone marrow-derived macrophages, macropinosomes in EGF-treated A431 cells have a different fate and do not fuse with the lysosomes. After approximately 5 min of EGF stimulation, macropinosomes in A431 cells become positive for transferrin receptors as well as for early endosomal antigen 1 (EEA1) (West, Bretscher et al. 1989; Hamasaki, Araki et al. 2004). Interestingly, macropinosomes in A431 cells do not mature beyond the early endosomal stage as indicated by the presence of EEA1. Instead, EEA1 mediates homotypic fusion between macropinosomes. Finally, the macropinosome fuses with the plasma membrane, recycling its content to the extracellular space (Hamasaki, Araki et al. 2004; Araki, Hamasaki et al. 2006).

Combined the above observations suggest that macropinosomes represent internalized membrane particles in membrane ruffles that are endocytosed upon pronounced membrane and cytoskeletal activity during cell activation and motility. Furthermore, macropinocytic processes differ depending on the cell type, therefore the basis for the different fates of macropinosomes in various cell types still remains to be determined.

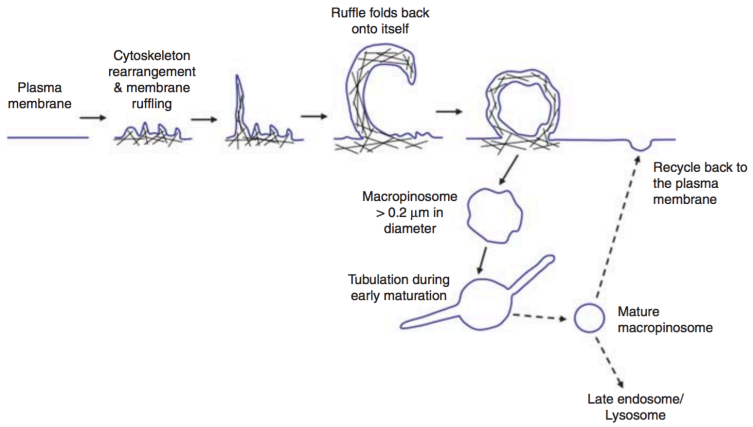

Figure 19: Macropinocytosis pathway. Macropinocytosis involves actin cytoskeleton rearrangement at the plasma membrane leading to the formation of membrane ruffles. Ruffles may fold back onto themselves and fuse at the base of plasma membrane, trapping solute and soluble substances in macropinosomes. Early maturation of macropinosomes involves extensive tubulation resulting in mature macropinosomes that are more spherical. The contents of the macropinosomes are then either degraded at the late endosome/lysosome or recycled back to the plasma membrane. The cytoskeleton is depicted as black lines (Lim and Gleeson 2011).

Clathrin/Caveolae-independent (CCI) endocytosis

This particular mode of endocytosis depends on the presence or absence of Dynamin.

Dynamin-dependent CCI

Dynamin-dependent CCI has been detected during the internalization of the β-chain of the interleukin-2 receptor (IL-2R-β). Interestingly, the specific endocytic pathway was not inhibited upon interference with clathrin-mediated endocytosis components. However, IL-2R-β uptake was strongly inhibited upon inhibition of dynamin-2 and RhoA, suggesting that RhoA - a key player in the regulation of actin dynamics - could possibly be required for the recruitment of the actin machinery to regulate endocytosis via this pathway (Gesbert, Sauvonnet et al. 2004).

Dynamin-independent CCI

Dynamin-independent CCI was first detected in HeLa cells, where the expression of a mutant dynamin-1 blocked receptor-mediated endocytosis whereas it increased fluid-phase uptake through clathrin-independent endocytosis (Damke, Baba et al. 1995). A common characteristic of constitutive dynamin-independent CCI endocytosis appears to be the involvement of small GTPases, either Arf6, or CDC42.

The role of CDC42 in dynamin-independent endocytosis was first described while studying the effects of a Rho GTPase inhibitor, Clostridium difficile toxin B (toxin B), or CDC42 dominant negative mutant on the internalization of Glycosylphosphatidylinositols-anchored proteins (GPI-APs). Interestingly, this dynamin-independent pathway seems to be the main route for the non-clathrin, non-caveolar uptake of cholera toxin B (CtxB) (Fivaz, Vilbois et al. 2002; Sabharanjak, Sharma et al. 2002).

ARF6 has been suggested to mediate dynamin-independent CCI endocytosis of several proteins such as class I major histocompatibility complex molecules (MHC I), β1 integrin, carboxypeptidase E (CPE), E-cadherin and GPI-APs (Naslavsky, Weigert et al. 2004; Powelka, Sun et al. 2004).

Nevertheless, although the exact factors that induce and promote the above undefined endocytic phenomena still remain unknown, their existence argues for the existing modes of endocytosis.

1.5.6 Endocytosis link to cell transformation

The identity of the normal, polarized epithelial cell is fundamentally tied to its ability to establish an intact mucosal sheet with the directional flow of ions, nutrients and receptor-dependent signals. A polarized epithelium requires the maintenance of apical and basolateral membranes with distinct characteristics and segregation of functional receptors such as channels, transporters, receptors and adhesion molecules in defined apical and basolateral zones separated by intercellular adherens junctions and tight junctions (Yeaman, Grindstaff et al. 1999). Continually self-renewing epithelia must maintain barrier function during renewal, which is supported by dynamic intracellular vesicle trafficking pathways that are responsible for the turnover of polarized membrane domains. Furthermore, in order to mediate the exchange of nutrients and crucial proteins, internalized proteins of epithelial cells may be transcytosed to the opposite surface (basolateral to apical and vice versa) (Fig. 20).

A better understanding of the above processes was enabled by the identification of epithelial cell channels and transporter proteins as well as apical structures such as microvilli and primary cilia (Nachury, Loktev et al. 2007; Muller, Hess et al. 2008; Feng, Knodler et al. 2012), which are necessary for maintaining all aspects of the normal epithelial physiology, including barrier functions, the presentation of enzymes and transporters, and the function of mucosal surfaces. Therefore, deregulated vesicle trafficking can greatly affect the physiology of the epithelial cell.

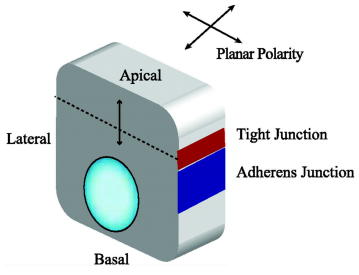

Figure 20: Basic model of epithelial cells. Epithelial cells arrange themselves in monolayers and are connected by junctions. Tight junctions (red) delineate apical vs. basolateral surfaces, while adherens junctions (blue) adhere cells to each other. Planar polarity is found perpendicular to the plane of apicobasal polarity.

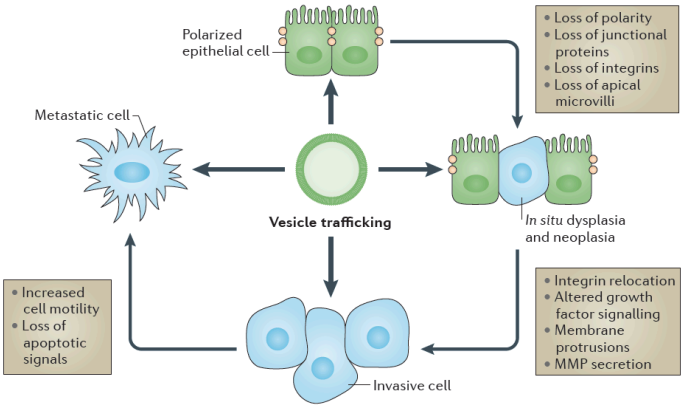

Figure 21: Vesicle trafficking plays key role during epithelial transformation. Vesicle trafficking is a central contributor for the evolution of epithelial cancers. The early loss of cell polarity is a crucial factor in early malignant changes. These changes include inappropriate trafficking of junctional proteins and cell adhesion molecules (for example, integrins, E-cadherin). Similarly, progression to a more invasive phenotype is associated with trafficking into membrane protrusions, the relocation of integrins, and the targeted secretion of matrix metalloproteinases (MMPs) at the invasive front. Finally, metastatic lesions are associated with further alterations in vesicle trafficking that promote cell motility and lead to decreased apoptosis (Goldenring 2013).

Alterations in the production or plasma membrane delivery of crucial regulators of structural polarity, including components of the intercellular junctions or cell adhesion molecules, influence polarized epithelial cell identity. Studies in Drosophila melanogaster have demonstrated that alterations in endocytic trafficking can lead to disruption of normal polarized cell and tissue development, and result in tumor formation (Morrison, Dionne et al. 2008).

Notably, some of the mutations that can promote endocytosis-induced invasive phenotype include E-cadherin mutations in familial gastric cancer (Guilford, Hopkins et al. 1998), APC mutations in familial polyposis (Oshima, Oshima et al. 1995), and BRCA1 mutations in breast cancer (Ashworth, Bertwistle et al. 1999). However, even in the case of those hereditary cancer mutations, there are further genetic or environmental perturbations required to cause cancer dissemination such as the ones mentioned below:

1. Aberrant trafficking (presentation or release) of a receptor ligand at the incorrect cell surface may lead to inappropriate signaling (Singh, Bogatcheva et al. 2013).
2. Inappropriate trafficking may cause the redistribution of cell adhesion molecules, such as integrins. Since integrin signaling is linked with cell proliferation and motility responses, these alterations can promote a transformed phenotype (Kuwada and Li 2000; Muller, Caswell et al. 2009; Arjonen, Alanko et al. 2012; Rainero, Caswell et al. 2012).
3. Altered delivery of junction components could elicit changes in the trans-epithelial permeability, and redistribute proteins that are normally segregated to apical or basolateral domains (Yokota, Ishii et al. 2000; Lioni, Brafford et al. 2007; Krishnan, Lapierre et al. 2011; Hayashi, Tamura et al. 2012). These changes can lead to aberrant signaling and promote a transformed phenotype.
4. Losses in cell polarity can lead to inappropriate delivery of degradative enzymes such as matrix metalloproteinases (MMPs) to cell surfaces, thereby promoting cell invasion and transformation (Williams and Coppolino 2011).

All of the above scenarios indicate that proper control of vesicle trafficking pathways is central to the maintenance physiological cell functions, and that alterations can contribute to epithelial transformation and tumor progression.

1.5.7 Endocytosis promotes epithelial to mesenchymal transition (EMT)

A hallmark of most solid tumors is the loss of epithelial cell markers and concomitant acquisition of mesenchymal cell markers, a profound phenotypic conversion referred to as epithelial to mesenchymal

transition (EMT). EMT phenotypes are observed in many epithelial tumors, such as non-small cell lung cancer (NSCLC), pancreatic, colorectal, and hepatocellular cancers particularly at the invasion front (Thiery and Sleeman 2006; Keshamouni and Schiemann 2009). EMT is orchestrated by the integration of growth factor cues, adhesion signaling pathways that control cytoskeletal morphology and gene expression profiles.

A critical molecular event favoring the disassembly of cell–cell contacts during EMT is the loss of the cell adhesion molecule, E-cadherin, a key component of the adherens junctions (Thiery and Sleeman 2006). EMT and metastatic progression are most often associated with a reversible downregulation of E-cadherin (encoded by CDH1) involving either hypermethylation of the CDH1 promoter or repression by EMT-inducing transcription factors (Thiery and Sleeman 2006; Berx and van Roy 2009). In particular, EMT is accompanied by the activation of two transcription factors, Snail and Slug. Notably, however, in addition to transcriptional downregulation, posttranscriptional regulation of adhesive structures can also markedly influence the progression of EMT (D'Souza-Schorey 2005). The endocytosis and lysosomal degradation of E-cadherin is one such cellular mechanism that can have a profound impact on the initial stages of EMT. Interestingly, the cytoplasmic domain of E-cadherin contains a dileucine motif, which is a binding site for clathrin adaptor complexes, and mutations in the motif can inhibit E-cadherin endocytosis (Miyashita and Ozawa 2007).

Growth factors such as HGF can also induce epithelial to mesenchymal transition (EMT) by promoting the endocytosis of E-cadherin molecules (Cox, Hengst et al. 2008; Wu and Hirsch 2009). More specifically, HGF can either trigger Arf6 activation, which leads to E-cadherin endocytosis (Palacios and D'Souza-Schorey 2003), or induce Ras-mediated activation of RIN2, an upstream activator of Rab5, which also results in E-cadherin endocytosis (Kimura, Sakisaka et al. 2006).

Inerestingly, Arf6 seems to be a critical regulator of E-cadherin internalization, since sustained Arf6 activation in 3D basement membrane cultures of epithelial cysts led to the internalization of E-cadherin as well as of growth factor receptors (Tushir, Clancy et al. 2010).

1.5.8 Endocytosis and cell invasiveness

Aberrant vesicle trafficking can result in enhanced cell migration. Generally, cell migration through a matrix requires the dynamic extension of cell protrusions, which are driven by the active turnover of cytoskeletal elements and the delivery and recycling of membranes and integral membrane proteins. Many components of the endocytic and recycling machinery are concentrated in the leading edges of invading cells (Hattula, Furuhjelm et al. 2006). Thus invasive cells are ‘’specialized’’ in their capability to directly deliver and recycle membrane vesicles to the invasion front. Knowledge of how these alterations occurs and how are maintained by vesicle trafficking pathways, is fundamental to understand the regulation of a cellular metastatic phenotype, which is critical for metastatic dissemination. Towards this end, the activity of multiple molecules that have been associated with endocytic activity (e.g. Rab proteins, MMPs, integrins) has been tested in the context of cellular trafficking-dependent cell invasiveness.

Rab proteins

Several studies have highlighted the role of Rab proteins in the regulation of cancer cell invasion, since overexpression of various Rab small GTPases and their effectors can result in invading structures and increase of cell migration (Zhang, Liu et al. 2009; Beaumont, Hamilton et al. 2011; Peranen 2011).

Two-pore channels

Recent studies have reported two-pore channels to be candidate targets for the treatment of metastatic cancers. It has been shown that genetic or pharmacological blockage of the two-pore channel TPC2, a key player in the regulation of endocytic transport, reduces lung metastasis in mammary mouse

cancer cells. Disruption of TPC2 function blocked trafficking of β1 integrin, leading to its accumulation in EEA1-positive early endosomes. Therefore, invasive cancer cells were no longer capable of forming leading edges, which are required for efficient migration. These findings highlighted the critical function of NAADP- and PI(3,5)P2-sensitive Ca2+permeable cation channels in the endolysosomal system of cells, and further support their potential as candidate targets for the treatment of invasive cancers (Ong Nam Phuong Nguyen1, Carina Atzberger1 et al. 2017).

Matrix degrading enzymes

Directed secretion of matrix-metallo proteases (MMPs) is a prerequisite for mesenchymal cell invasion within a matrix. Interestingly, this process seems to be mediated by endo- and exocytosis, since it has been reported that Rab8 along with the SNARE protein vesicle-associated membrane protein 7 (VAMP7) mediate the secretion of MMP14, which regulates the invasive phenotype of HeLa and osteosarcoma cells, indicating that the endocytosis-dependent release of degrading enzymes increases cell migration levels (Bravo-Cordero, Marrero-Diaz et al. 2007; Steffen, Le Dez et al. 2008; Poincloux, Lizarraga et al. 2009).

Integrins

A critical aspect of cell migration is the redistribution of adhesion receptors, particularly integrins. Migrating cells display enhanced integrin internalization and recycling from the retracting edge of the cell to the leading edge and at sites of cell invasion. Interestingly, these trafficking events regulate the formation of invasive structures such as filopodia and invadopodia (Caswell, Spence et al. 2007; Poincloux, Lizarraga et al. 2009). Moreover, integrins are redistributed during cell migration from focal adhesions that disassemble at the cell's trailing edge towards new focal adhesions that assemble at the leading edges. Therefore, modifications in integrin recycling, activation and/or presentation on the cell surface can markedly alter the invasive behaviour of cancer cells (Muller, Caswell et al. 2009; Mai, Veltel et al. 2011; Rainero, Caswell et al. 2012). Indeed, several reports have described the association of integrin trafficking with an increase of migratory potential in several tumor cells lines (Ramsay, Keppler et al. 2007; Ramsay, Marshall et al. 2007). In addition, integrin turnover is also thought to facilitate the uptake of ECM proteins such as fibronectin and vitronectin and transport of these molecules to the lysosomes (Shi and Sottile 2008; Lobert, Brech et al. 2010).

Integrins can be endocytosed either through clathrin-dependent or clathrin-independent mechanisms: Integrin receptor localization was detected in clathrin-coated pits (De Deyne, O'Neill et al. 1998), and there are studies indicating that integrins can be internalized through macropinocytosis-dependent mechanisms (Gu, Noss et al. 2011). Interestingly, regarding the latter, it has been shown that during growth factor-induced cell migration integrins are redistributed to dorsal circular ruffles and are internalized by macropinocytosis. The internalized integrins then transit to endosomal compartments to repopulate newly formed focal adhesions on the ventral surface (Gu, Noss et al. 2011).

Since dynamic integrin turnover is central to cell migration, integrin association with Rab endosomal proteins are likely to be important for integrin signaling and of course in cell migration. Interestingly, genetic interference with Rab proteins impeded integrin-mediated cell adhesion and motility, indicating that integrin-mediated cell migration relies on proper integrin endosomal sorting (Pellinen, Arjonen et al. 2006). Furthermore, Rab5 and Rab21 are associated with the plasma membrane and early endosomes and regulate the internalization of β1 integrins via direct interaction with their α subunits (Pellinen, Arjonen et al. 2006). Reduction of Rab5 or Rab21 in carcinoma-associated fibroblasts decreased α5 integrin at the plasma membrane, which enhanced the invasion of squamous cell carcinoma. Additionally, it has been shown that Rab25 directly binds to the β1 subunit of $\alpha_5\beta_1$ integrin and facilitates its recycling to the leading edge promoting invasiveness (Caswell, Spence et al. 2007). Finaly, Rab11 controls $\alpha_6\beta_4$ integrin recycling involved in hypoxia-induced breast cancer cell invasion (Yoon, Shin et al. 2005).

Notably, altered expression has been reported for various Rab proteins that control integrin trafficking, while those altered experession levels are often associated with various types of cancer. For

instance, Rab5 is highly expressed in lung adenocarcinoma and hepatocellular carcinoma (Fukui, Tamura et al. 2007), while overexpression of Rab25 is well documented in many types of cancers including ovarian cancer, breast cancer, testicular tumor, Wilms tumor, and bladder carcinomas (Li 2011).

Arf6, the primary mediatiator of macropinocytosis has also been reported to control integrin trafficking towards cell migration. More specifically, it has been found that Arf6 and β1 integrin associate in Arf6-regulated recycling endosomes, and the expression of a dominant negative form of ARF6 abrogated Arf6-dependent recycling, leading to a significant decrease of integrin receptor recycling towards the leading edge (Brown, Rozelle et al. 2001; Powelka, Sun et al. 2004).

In addition, GEP100, which is an Arf6 GEF, as well as ACAP1, which is an Arf6 GAP, also regulate integrin trafficking: GEP100, which is upregulated in breast cancers, has been implicated in the trafficking of β1 integrin receptors (Dunphy, Moravec et al. 2006; Sabe, Hashimoto et al. 2008), while ACAP1 activity was shown to be crucial for integrin recycling and cell migration (Li, Ballif et al. 2005).

Thus the endocytic machinery consists of a complex network of signaling molecules and adaptors that is critical for many cellular functions such as internalization, recycling and redistribution of nutrients and receptors. However, apart from the prominent role of endocytosis in the context of import and recycling, the above findings indicate that it also contributes to orchestrating the framework of signaling pathways controlling cell invasion and metastasis. Thus, the endocytic machinery not only controls expression and function of surface receptors involved in migration and invasion, but also enables their downstream signaling, which combined can contribute to the metastatic phenotype.

1.6 Integrins

Integrins consist a family of cell surface transmembrane receptors, responsible for cell-ECM and cell-cell adhesion. Due to their functions in tissue organization, integrins are considered critical molecules for multicellular organism development (Tamkun, Desimone et al. 1986; Hynes 1987; Hynes 1992).

Integrins function by promoting cell adhesion, cell survival, differentiation, growth and are essential for connecting the intracellular with the extracellular space, leading to cytoskeleton arrangements (Cabodi, Di Stefano et al. 2010; Nistico, Di Modugno et al. 2014; Iwamoto and Calderwood 2015). These functions are fundamental during embryonal development and wound healing, as well as in various pathologies. Integrins are the main components of adhesion force generation, crucial mediators of mesenchymal-like and collective migration, both of which are relevant in cancer (Schmidt and Friedl 2010).

1.6.1 Structure

Integrins are heterodimeric receptors composed of two non-covalently associated α (eighteen isoforms) and β (eight isoforms) glycoprotein subunits. Different combinations of α and β subunits (24 different types of human integrins) provide integrin receptors with different affinities for ECM molecules. Both subunits span the cell membrane, with short intracellular C-terminal tails and large N-terminal extracellular domains (Fig. 22). The extracellular domains bind to specific amino acid sequence motifs in extracellular matrix proteins or, in some cases, in proteins on the surfaces of other cells (Hynes 1992).

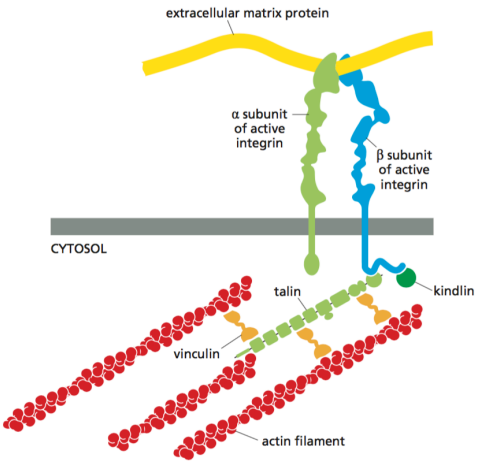

Figure 22: The structure of an active integrin molecule, linking the extracellular matrix to the actin cytoskeleton. The N-terminal heads of the integrin chains attach directly to an extracellular protein such as fibronectin. The C-terminal intracellular tail of the integrin β subunit binds to adaptor proteins that interact with cortical actin. Talin is the best-characterized protein adaptor and contains a string of multiple domains for binding actin and other proteins, such as vinculin, which help reinforcing and regulating the linkage to cortical actin filaments. One end of talin binds to the cytoplasmic tail of the β subunit, whereas other regulatory proteins, such as kindlin, bind at another site on the tail (Alberts, Johnson et al. 2015).

The best-characterized recognition site for integrins is the tripeptide Arg-Gly-Asp (RGD) sequence, which is found in fibronectin and other extracellular matrix proteins. Additional integrin-binding sequences, as yet poorly defined, exist in laminins and collagens (Kanchanawong, Shtengel et al. 2010; Ross, Coon et al. 2013).

The intracellular portion of an integrin dimer causes the assembly of a multiprotein complex, which together establish the linkage to the F-actin cytoskeleton (Fig. 22). The large adaptor protein talin is a component of the linkage in many cases, but numerous additional proteins are also involved. An example of the actin-linked cell–matrix junctions formed by integrins, are the focal adhesions that form when fibroblasts establish strong attachments to the rigid surface of a culture dish, as well as the myotendinous

junctions that attach muscle cells to their tendons. In epithelia, the most prominent cell–matrix attachment sites are the hemidesmosomes, where a specific type of integrin anchors the cells to laminin in the basal lamina (Moser, Legate et al. 2009; Hoffman, Grashoff et al. 2011; Calderwood, Campbell et al. 2013).

1.6.2 Integrin activation

Interaction of the integrin extracellular head domain with the ECM ligand or divalent cations leads to partial unfolding of the extracellular domain and separation of integrin subunits, which leads to the activation of the integrin heterodimer. Integrin molecules have no kinase activity. However, multiprotein complexes assembly, which results in the activation of intracellular signaling that regulates cellular shape and functions. This process is called ‘outside-in’ signaling (Campbell and Humphries 2011).

Structural studies suggest that integrins exist in multiple conformations associated with different states of activity (Fig. 23) (Dong, Mi et al. 2012). In the inactive state, the external segments of the integrin dimer are folded together into a compact structure that cannot bind matrix proteins. In this state, the cytoplasmic tails of the dimer are hooked together, preventing their interaction with cytoskeletal linker proteins. In the active state, the two integrin subunits are unhooked at the membrane to expose the intracellular binding sites for cytoplasmic adaptor proteins, and the external domains unfold and extend, to expose a high-affinity matrix-binding site at the tips of the subunits. Thus, the switch from inactive to active states depends on a major conformational change that exposes the external and internal ligand-binding sites at the ends of the integrin molecule. External matrix binding and internal cytoskeleton linkages are thereby coupled (Tamkun, Desimone et al. 1986; Luo and Springer 2006; Shattil, Kim et al. 2010).

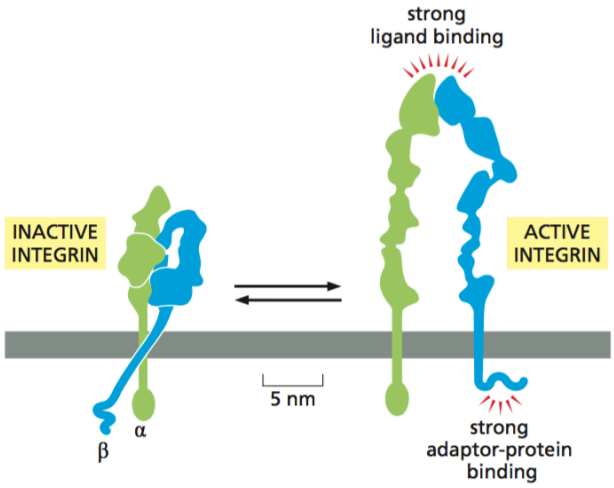

Figure 23: Integrins exist in two major activity states. Inactive (folded) and active (extended) structures of an integrin molecule, based on data from x-ray crystallography and other methods (Takagi, Petre et al. 2002).

Switching between the inactive and active states is regulated by a variety of mechanisms. Activation can occur by an “outside-in” mechanism whereby the binding of an external matrix protein, such as the RGD sequence of fibronectin, can drive some integrins to switch from the low-affinity inactive state to the high-affinity active state. As a result, binding sites for talin and other cytoplasmic adaptor proteins are exposed at the tail of the β chain. The binding of these adaptor proteins then leads to the attachment of actin filaments to the intracellular end of the integrin molecule. In this way, when the integrin binds its ligand outside the cell, the cell reacts by tying the integrin molecule to the cytoskeleton, so that force can be applied at the point of cell attachment (Shattil, Kim et al. 2010).

Inversely, the cause and effect can also operate from inside to outside. This “inside-out” integrin-activation process generally depends on intracellular regulatory signals that stimulate the ability of talin and other proteins to interact with the β chain of the integrin. Talin competes with the integrin α chain for

its binding site on the tail of the β chain. Thus, upon talin binding to the β chain, α–β linkage is blocked, allowing the two subunits of the integrin molecule to disintegrate (Calvete 2004).

1.6.3 Integrin signaling

One of the best-studied mechanisms of integrin signaling depends on a cytoplasmic protein tyrosine kinase called focal adhesion kinase (FAK). Focal adhesions are sites of prominent tyrosine phosphorylation, with FAK being one of the major tyrosine-phosphorylated proteins involved. When integrins cluster at cell–ECM contacts, FAK is recruited to the integrin β subunit by intracellular adaptor proteins such as talin or paxillin (Fig. 24). The clustered FAK molecules phosphorylate each other on the tyrosine residues, creating a phosphotyrosine docking site for members of the Src tyrosine kinases (Schlaepfer and Hunter 1997). Phosphorylated FAK can also phosphorylate phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), phospholipase Cγ (PLCγ), and growth factor receptor bound protein 7 (Grb7), leading to their activation (Guo and Giancotti 2004; Reardon, Neyns et al. 2011). Therefore, outside-in signaling from integrins via FAK and Src family kinases, is relayed into the cell in a way similar to the signaling pattern generated from receptor tyrosine kinases.

The Src/Fak complex can also lead to the phosphorylation of p130CAS and paxillin, promoting the formation of a protein complex with Crk and Crk-dedicator of cytokinesis (Dock) (Reardon, Neyns et al. 2011). The protein complex leads then to the activation of Rac. Rac further activates p21-activated kinase (PAK), Jun amino-terminal kinase (JNK), and nuclear factor κB (NF-κB), and PI3K to AKT/protein kinase B (PKB). Furthermore, the activated Fak/Src complex can recruit Grb2, which promotes the formation of a complex containing Fak and Grb2/Shc and subsequently activates the ERK/MAPK pathway (Guo and Giancotti 2004).

Notably, once FAK is activated in focal adhesions, it promotes actin dynamics by recruiting Arp2/3 in the lamellipodium (Serrels, Lund et al. 2015; Swaminathan and Waterman 2016). Subsequently, β-PIX and the kindlin–paxillin complex further promote Arp2/3 activity through activating the Rac1 GTPase in focal adhesions (Kuo, Han et al. 2011; Theodosiou, Widmaier et al. 2016).

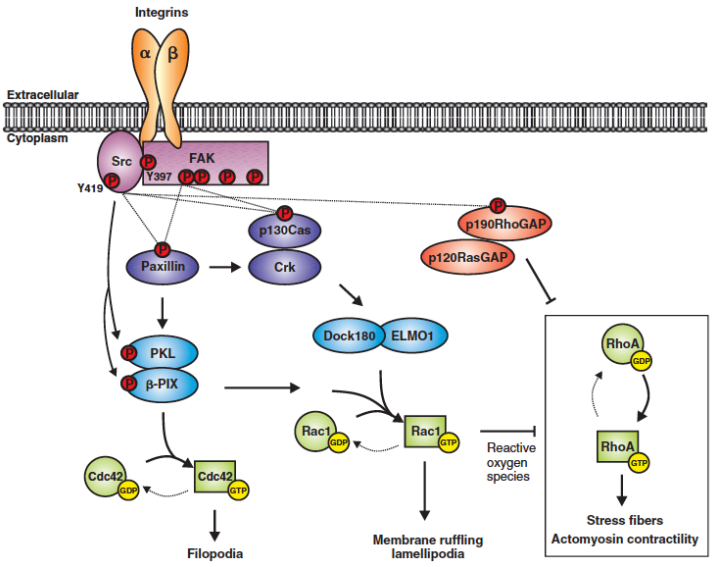

Figure 24: Integrin signaling. Integrin regulation during early stages of cell spreading. The FAK-Src complex activates several pathways that lead to protrusive activity via Rac and Cdc42 GTPases at sites of integrin ligation. At the same time, this complex, mediates suppression of actomyosin contractility by keeping the activity of RhoA low. Broken lines indicate protein-protein interactions (Huveneers and Danen 2009).

1.6.3.1 Integrin-RTK crosstalk

The most compelling evidence of integrin-RTK crosstalk derives from their direct, physical association. Co-immunoprecipitation assays have confirmed those two receptor interactions. For instance, $\alpha_v\beta_3$ integrin associates with insulin-like growth factor receptor (IGFR-1), platelet-derived growth factor receptor (PDGFR) (Schneller, Vuori et al. 1997; Borges, Jan et al. 2000), and vascular endothelial growth factor receptor-2 (VEGFR2) (Soldi, Mitola et al. 1999). Additionally, $\alpha_6\beta_4$ integrin has been shown to associate with ErbB2 (Falcioni, Antonini et al. 1997), c-Met (Trusolino, Bertotti et al. 2001), EGFR (Mariotti, Kedeshian et al. 2001), and macrophage-stimulating protein receptor (Santoro, Gaudino et al. 2003). These interactions suggest that signaling crosstalk between integrins and RTKs may be the result of receptor co-clustering upon cell adhesion or growth factor stimulation. Growth factor stimulation of RTKs or ECM-integrin interactions induces an increase in the local concentration of integrins and RTKs at focal adhesions and at leading edges of tumor cells, such that crosstalk could occur even without direct physical association (Lipscomb and Mercurio 2005). The mode of signaling cooperation between integrins and RTKs could be reciprocal as well as uni-directional. Bi-directional cooperation between the two signaling systems was demonstrated for the $\alpha_v\beta_3$-IGF receptor (Maile and Clemmons 2002) and $\alpha_v\beta_3$-PDGF- complex (Baron, Shattil et al. 2002), whereupon signaling pathways activated by both receptors converged downstream at common signaling effectors. Uni-directional, integrin dependent RTK activation was demonstrated for β1 integrin-dependent EGFR activation. β1 integrin activation leads to the c-Src dependent recruitment of EGFR and p130Cas, and subsequently to phosphorylation and activation of EGFR in an EGF-independent manner (Moro, Dolce et al. 2002).

1.6.3.2 Integrin signaling and P53

Notably, integrin signaling has been shown to induce p53 activity leading to the induction of chemotherapy resistance. It has been shown that α5β1 integrin impairs chemotherapy-induced p53 activation (Martinkova, Maglott et al. 2010; Janouskova, Maglott et al. 2012; Janouskova, Ray et al. 2013). Similar findings were obtained in breast carcinoma cells revealing enhanced expression of p53 upon deletion of $\alpha_2\beta_1$ (Morozevich, Kozlova et al. 2012). In high-grade glioma, high expression of α5 subunit inhibited the temozolomide-induced p53 pathway, inducing resistance to chemotherapy (Janouskova, Maglott et al. 2012).

It has been reported that FAK can inhibit p53 upon direct interaction in the nucleus or cytoplasm (Golubovskaya and Cance 2011; Golubovskaya 2014), suggesting that integrin signaling affects p53 function. Additionally, impairment of α5 integrin or FAK signaling in combination with activation of p53 results in tumor cell apoptosis (Gillory, Stewart et al. 2015; Renner, Janouskova et al. 2016). Therefore, blocking either integrins or their downstream signaling pathways may offer new opportunities to synergistically enhance the p53 tumor suppressor effects.

1.6.4 Integrin Functions

Depending on the surrounding matrix, integrins lead to the activation of intracellular signaling cascades thereby controlling many aspects of cellular behavior. Hence, aberrations in integrin regulation/expression, or mutations in the major integrin effectors can lead to various disorders, examples of which are listed below (Wehrle-Haller and Imhof 2003).

1.6.4.1 Embryonic development

In vertebrate embryos, integrins are required for neural crest migration. In chicken, the major players in this process are the $\alpha_4\beta_1$ and $\alpha_1\beta_1$ integrins, which can bind to fibronectin and laminin-1, respectively (Desban and Duband 1997; Kil, Krull et al. 1998). Initiation of $\alpha_4\beta_1$ expression coincides with the beginning of neural crest cells migration, and inhibition of the interaction between $\alpha_4\beta_1$ integrin and its ECM ligands with blocking antibodies, ligand-mimicking peptides, or antisense oligonucleotides leads to a

severe reduction both in the number of migrating cells and the distance covered (Testaz and Duband 2001)

Integrins $\alpha_5\beta_1$ and $\alpha_v\beta_5$ are involved in the renewal, survival, and differentiation of human embryonic stem cells. Moreover, the α5 integrin subunit, as well as fibronectin, play critical roles in the regulation of blood vessel development in mouse embryos, since deletion of α5 integrin leads to the development of considerably distended blood vessels (Francis, Goh et al. 2002). Notably, integrin α3 knockout mice are perinatally lethal, with marked abnormalities in lung development (Kreidberg, Donovan et al. 1996).

1.6.4.2 Immune function

Serious immune disorders can be caused by integrin defects: Integrin aberrant signaling can lead to leukocyte adhesion deficiency (LAD), which is characterized by the inability of leukocytes to adhere and migrate during inflammatory and host defense reactions (Etzioni 2007). As a result, patients with this autosomal recessive syndrome present with repeated bacterial and fungal infections. In resting leukocytes and platelets, the integrins are held in a bent/inactive conformation that limits their ability to interact with ligands (endothelial ICAMs or extracellular matrix proteins such as fibrinogen or collagen). Following cellular activation (by agonists including chemokines, growth factors, antigens or selectin ligands), integrins are activated and inside-out signaling occurs. LAD patients show complete failure to activate β1 and β2 integrins on leukocytes, as well as β3 integrins on platelets (Kilic and Etzioni 2009). Of note, impaired integrin signaling derived from mutations in the kindlin gene has been confirmed as the leading cause of LAD. Due to those mutations, kindlin fails to be recruited to integrins, therefore integrin inside-out signaling is disrupted. Hence, kindlin plays a central role in the activation of hematopoietic integrins, since it allows leukocyte adhesion/migration in response to infection and platelet activation for normal thrombosis (Ussar, Moser et al. 2008; Moser, Bauer et al. 2009).

Numerous autoimmune disorders have been characterized by an imbalance or upregulation of angiogenic processes. Best known are rheumatoid arthritis (Storgard, Stupack et al. 1999), psoriasis (Creamer, Sullivan et al. 2002), restenosis (Bishop, McPherson et al. 2001), diabetic retinopathy (Chavakis, Riecke et al. 2002) and tumour growth (Folkman 2002). Moreover, a variety of other allergic, inflammatory, traumatic, infectious, metabolic and hormonal disorders are characterised by up-regulated vessel growth (Carmeliet 2003). $\alpha_v\beta_3$ integrin is a receptor with a prominent role in angiogenesis, since it mediates the migration of endothelial cells through the basement membrane during blood vessel formation. Notably, $\alpha_v\beta_3$ integrin expression is found not only in endothelial cells during tumour-induced angiogenesis, but also during wound healing, in rheumatoid arthritis, in psoriatic plaques and during restenosis.

Integrins are also implicated in age-related macular degeneration (AMD), the leading cause of blindness in the elderly population. AMD is characterized by neovascularization of the Retinal Pigmented Epithelium (RPE) (Klein and Francis 2003). Integrin $\alpha_v\beta_5$ is localized at the apical RPE and binds to ligands in the interphotoreceptor matrix (IPM), participating in the interactions between photoreceptors and the RPE (Mallavarapu and Finnemann 2010). In $\alpha_v\beta_5$ integrin-deficient mice, retinal adhesion and phagocytosis of photoreceptor outer segments are compromised (Nandrot, Kim et al. 2004). It has been revealed that $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins (Friedlander, Theesfeld et al. 1996; Luna, Tobe et al. 1996) were expressed in neovascular ocular tissue from patients with wet AMD. Hammes and colleagues showed that subcutaneous injection of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ antagonists remarkably prevented retinal neovascularization in a mouse model of hypoxia induced proliferative retinopathy (Hammes, Brownlee et al. 1996). These results indicate the possibility that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins might be a therapeutic target for AMD. Additionally, the effectiveness of $\alpha_v\beta_1$ and $\alpha_v\beta_5$ integrin antagonists (JNJ-26076713) against ocular neovascularization has been well documented (Santulli, Kinney et al. 2008).

Interestingly, immune cell function regulation including T-cell activation and lymphocyte adhesion to endothelium through transforming growth factor-β (TGF-β) depends on $\alpha_v\beta_6$ and $\alpha_v\beta_8$ integrins (Worthington, Fenton et al. 2012). Furthermore, both integrin $\alpha_4\beta_1$ and integrin $\alpha_L\beta_2$ have been associated with inflammatory conditions, particularly autoimmune diseases and asthma (Bao, Omana et al. 2012).

Integrins have been proposed to have fundamental role in activating TGFβ1 to control immune homeostasis. Knock-in mice expressing TGFβ1 with a point mutation in the RGD integrin binding site showed a remarkably similar phenotype to mice completely lacking TGFβ1 production, dying from multi-organ inflammation early in life (Yang et al. 2007). Subsequent work has shown that integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ are the key activators of TGFβ1 in the steady state immune system, as a combined lack of function of these integrins recapitulates the phenotype seen in TGFβ1-knockout mice (Aluwihare et al. 2009). Thus, integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ play a non-redundant role in the activation of TGFβ1 in the immune system which is required to prevent self-harmful immune responses.

1.6.4.3 Coagulation and Cardiovascular disease

Integrin $\alpha_{IIb}\beta_3$ is the fibrinogen receptor and has a prominent role in platelet aggregation and blood clotting. In parallel, integrin $\alpha_{IIb}\beta_3$ consists the main integrin type expressed on platelets cell surface. Upon platelet association with collagen, integrin $\alpha_{IIb}\beta_3$ changes shape, leading thus to fibrin binding, clot matrix formation and bleeding arrest (Niu, Chen et al. 2012). Due to $\alpha_{IIb}\beta_3$ essential role in platelet aggregation, its role as an antithrombotic factor has been established and $\alpha_{IIb}\beta_3$ antagonists (abciximab, eptifibatide, and tirofiban) have been approved. Importantly, the rational targeting of $\alpha_{IIb}\beta_3$ and the clinical efficacy of $\alpha_{IIb}\beta_3$ antagonists established the central role of platelets in periprocedural thrombosis in the context of percutaneous coronary interventions (PCI) (Bledzka, Smyth et al. 2013).

1.6.5 Integrins in cancer

The metastases spawned by carcinomas are formed following the completion of a complex succession of cell-biological events, collectively termed the invasion-metastasis cascade. Briefly these steps are listed below:

1. Local tissue infiltration through the degradation of the basement membrane
2. Intravasation into the blood vessel lumen,
3. Adhesion at the vessel well
4. Survival of the rigors of transport through the vasculature
5. Arrest at distant organ sites,
6. Extravasation into the parenchyma of distant tissues
7. Establishment of metastatic microenvironment (niche)
8. Outgrowth (Fidler 2003)

Since integrins are implicated in many of the stages of the invasion-metastasis cascade, it is of no surprise that increased expression of certain integrin subunits has been reported for Cancer Stem Cells (CSCs) and has been linked with poor prognosis and increased metastasis in a variety of cancers (Desgrosellier and Cheresh 2010; Medema 2013) (Fig. 25). Some of the integrin-attributed functions enabling the initiation of various steps responsible for metastatic progression are described below.

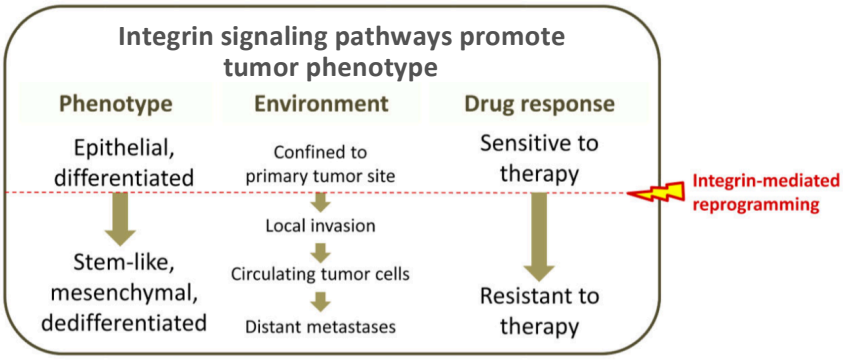

Figure 25. Reprogramming of cancer cells by integrin signaling pathways: Integrin signaling is capable of reprogramming tumor cells to promote invasion, hematogeneous dissemination, and establishment at distant metastatic sites. Similarly, stemness and drug resistance are influenced by integrin-mediated cell-matrix adhesion and the underlying signaling. Understanding these events offers new therapeutic opportunities for cancer (Seguin, Desgrosellier et al. 2015).

1.6.5.1 Anchorage-independent growth

In order to grow, proliferate and survive, most cell types (e.g. epithelial, endothelial, muscle cells) depend on the attachment on a substratum, whereas upon loss of contact with the extracellular matrix, they undergo apoptosis. This form of apoptosis is called anoikis and results from the lack of the integrin-dependent pro-survival signaling pathways (e.g. PI3K/AKT, MEK/ERK, FAK, NFκB) that manifests when cell adhesion decreases (Vachon 2006; Griffiths, Grundl et al. 2011). Mutations that disrupt or override this form of anchorage-dependent growth inhibition and make cells resistance to anoikis often occur in cancer cells, promote tumor progression and favor the emergence of metastasis (Paoli, Giannoni et al. 2013; Buchheit, Weigel et al. 2014). When tumor cells escape from the primary tumor and intravasate into the blood or lymphatic vessels, they need to survive in the absence of adhesion to ECM. Mounting evidence supports a pivotal role for aberrant integrin signaling in controlling growth and survival when no anchorage is available, a property critical for hematogeneous metastasis. For instance, integrin β1 mediates anchorage-independent growth in prostate (Schooley, Andrews et al. 2012) and breast cancer cells by activating a FAK/PAK/MAPK signaling pathways (Cagnet, Faraldo et al. 2014), whereas integrin β3 interacts with c-Src to suppress anoikis and promote lymph node metastasis in breast cancer as well as pancreatic tumor models (Desgrosellier, Lesperance et al. 2014).

In melanoma cells, TIMP1, an MMP inhibitor, interacted with CD63 and integrin β1 conferring resistance to anoikis (Toricelli, Melo et al. 2013). Additionally, zinc finger transcription factor ZNF304 was recently identidied as a novel transcriptional regulator of β1 integrin that promotes cancer cell survival, and protects against anoikis in ovarian cancer (Aslan, Monroig et al. 2015), while cilengitide, an $\alpha_v\beta_3/\beta_5$ integrin inhibitor, induced atypical anoikis involving necrosis and autophagy in glioma cells (Silginer, Weller et al. 2014). Furthermore, suppressed anoikis was recently attributed to enhanced integrin endosomal signaling (Alanko, Mai et al. 2015).

The above examples portray the different ways through which a cancer cell can acquire resistance to detachment-induced apoptosis through modulating integrin expression and functions.

1.6.5.2 Invasion/metastasis

Integrins have been implicated in all steps of tumor progression, including detachment and invasion of tumor cells, intravasation into lymphatic and blood vessels, survival and transport through the circulation, arrest in distant organs, extravasation into distant organs, and growth of secondary lesions. The initial steps of tumor dissemination from a primary site involve a variety of functions mediated and regulated by different integrin combinations (Weis and Cheresh 2013; Desgrosellier, Lesperance et al. 2014).

The capacity to dynamically interact with and respond to the surrounding tissue is an essential prerequisite of a cancer cell to migrate. The result of this interaction can be the phenotypic conversion known as the epithelial-to-mesenchymal transition (EMT). This process is critical during development but is also frequently triggered during cancer metastasis. EMT is characterized by the transition to a mesenchymal phenotype, involving the disassembly of cell-cell contacts, cytoskeletal reorganization, and acquisition of mesenchymal markers and migratory properties (Lamouille, Xu et al. 2014). It is of no surprise that the mechanisms critical for EMT, stemness and drug resistance demonstrate significant overlap (Singh and Settleman 2010), and it is likely that integrins play a critical role in allowing tumor cells to become more aggressive and therapy-resistant.

Matrix degradation is another cellular process that increases tumor cell invasion. Notably, integrins are known regulators of MMPs, facilitating ECM degradation and remodeling (Borrirukwanit, Pavasant et al. 2014; Missan, Mitchell et al. 2015). Additionally, integrins have also been shown to regulate

angiogenesis, thus contribute also to later stages of tumor development (Schaffner, Ray et al. 2013).

The crosstalk between integrins and GFs also accelerates tumor cell motility and invasion. Clinical and functional analyses showed that CD151 (member of the tetraspanin family) and $\alpha_3\beta_1$ integrin were elevated in glioblastoma. Both synergized with EGF/EGFR complex to accelerate tumor cell motility and invasion (Zhou, Erfani et al. 2015). Additionally, it has been demonstrated that fibronectin matrix mediates PDGFR-β association with $\alpha_5\beta_1$ integrin in focal adhesions, leading to enhanced cell migration (Veevers-Lowe, Ball et al. 2011). Moreover, HGF-mediated c-Met activation induces collective cancer cell invasion through β1 integrin trafficking (Mai, Muharram et al. 2014). Hence the above findings suggest that β1 integrin and GFs either synergistically act in parallel signaling pathways or share the same signaling pathways to modulate cancer cell migration.

1.6.5.3 Colonization of the metastatic niche

Metastatic colonization of distant organs requires the survival as well as the expansion of cancer cells at the secondary site. Since seeding a ‘’metastatic niche’’ requires specific recognition mechanisms between the cancer cells and their surrounding ECM, only a minority of cancer cells will eventually manage to reach the distant organ and accomplish the process. Since the integrin expression pattern determines the capability of a cancer cell to ‘’seed the niche’’ and initiate metastatic colonization, integrins may therefore be considered critical for the ‘’homing’’ of tumor cells into tissue environments that enhance metastasis.

Interestingly, the propensity for metastasis has been associated with the accumulation of certain ECM proteins within a particular metastatic niche. For instance, tenascin C, a ligand for β1 and β3 integrins that is produced within the lung metastatic niche, correlates with poor outcome in breast cancer patients (Oskarsson, Acharyya et al. 2011). Furthermore, the $\alpha_v\beta_3$ ligand L1-CAM is expressed in breast cancer cells and is required for breast cancer metastasis to the lungs, enabling tumor extravasation towards the lung endothelium (Zhang, Wong et al. 2012). Similarly, integrins $\alpha_v\beta_3$, $\alpha_2\beta_1$, and $\alpha_4\beta_1$ play critical roles in bone metastasis, as their ligands represent ECM proteins normally expressed in bone-associated cells (Esposito and Kang 2014). Furthermore, knock-out mice for $\alpha_{11}\beta_1$ integrin, a cell-specific receptor for fibrillar collagen overexpressed in carcinoma associated fibroblasts (CAF), abolished the metastatic potential of lung adenocarcinoma cells to bone, kidney, or brain (Navab, Strumpf et al. 2016). Cumulatively, the above findings portray the complexity of integrin-ligand binding events that govern the mechanisms during the formation of new micrometastatic colonies arising from tumor circulating cells.

Once entrenched within an appropriate metastatic niche, tumor cells must adapt again to survive and proliferate. Integrin β1-mediated filopodium-like protrusions that support the initial interactions between the extravasated cancer cells and ECM components of the host tissue induce the formation of filopodia-like protrusions that ultimately lead to the initiation of adhesion-dependent signaling events such as FAK phosphorylation and subsequent activation of ERKs, that result in rapid proliferation and spreading (Shibue and Weinberg 2009; Shibue, Brooks et al. 2012). Upon the occurrence of the aforementioned adhesion-dependent events, it is also likely that the expression of the integrins providing anchorage-independent growth has less influence over the adhesion-dependent growth while prevailing growth advantages for circulating cells. Since an integrin receptor can bind to multiple ligands, and a single ligand can recognize multiple integrin heterodimers, spatiotemporal regulation patterns of integrin vs. ligand expression ultimately determine how a cell senses and responds to its environment.

1.6.5.4 Integrin endosomal signaling and cell migration

Integrin function is not only achieved through ligand engagement and binding of intracellular proteins but also through integrin endocytic trafficking. Integrin trafficking offers an important complementary mechanism for regulating integrin–ECM adhesion turnover and controls many aspects of cellular behavior, including cytokinesis, cell motility, and invasion. Internalization pathways for integrins include clathrin-dependent as well as clathrin-independent mechanisms. These include macropinocytosis

from circular dorsal ruffles (CDRs) triggered by growth factor receptor signaling (Gu, Noss et al. 2011) as well as caveolae-mediated endocytosis (Bridgewater, Norman et al. 2012). There is clear evidence that integrin endocytosis comprises an important regulation mechanism for cell migration both in 2D and 3D (Caswell and Norman 2008; Valdembri and Serini 2012).

Focal adhesion turnover and cell migration control

Integrin trafficking has been linked to cell migration mainly through the turnover of focal adhesions/focal complexes (Webb, Parsons et al. 2002). Focal adhesions are essential components towards the regulation of cellular adhesion and migration. Integrins have to be trafficked from the rear to the front of the cell to assemble new focal adhesions. These newly formed adhesions are subsequently disassembled for cell detachment. These continuous cycles of focal adhesion assembly/disassembly are crucial for cell migration as they provide the cell with a constant pool of free integrins to engage the ECM and generate new adhesions (Scita and Di Fiore 2010; Morgan, Hamidi et al. 2013). Hence integrins need to be dynamically turned over, which is achieved by endocytic trafficking (Ridley, Schwartz et al. 2003; Maritzen, Schachtner et al. 2015).

Integrin association with cytoskeletal structures

Actin-binding proteins or nucleation/assembly factors have been reported to play crucial roles in the pro-invasive activity of integrins: FMNL2 (Formin-like 2-actin nucleation and assembly factor), which is upregulated in several aggressive cancers, interacts with RhoC to drive $\alpha_2\beta_1$ and $\alpha_5\beta_1$ integrin internalization/trafficking and invasive motility of cancer cells (Wang, Arjonen et al. 2015). Furthermore, microtubules mediate the delivery of the mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) to focal adhesions, which leads to enhanced focal adhesion dissolution through an Arf6-dependent mechanism (Yue, Xie et al. 2014). Additionally, microtubule-associated cytoplasmic linker associated proteins 1 and 2 (CLASP1 and CLASP2) have been shown to trigger FA disassembly and turnover through the exocytosis of MMPs (Stehbens, Paszek et al. 2014).

Another mechanism involving integrin trafficking in cell migration is the formation of invasive structures such as invadopodia and filopodia: Invadopodia are sites of active ECM degradation mainly by the type 1 matrix metalloproteinase (MT1-MMP, also known as MMP14) and consist a typical characteristic of invasive cells (Linder, Wiesner et al. 2011). On the contrary, filopodia are integrin-dependent structures implicated in cancer cell invasion (Arjonen, Kaukonen et al. 2014). Although invadopodia and filopodia share similarities, invadopodia-mediated cell invasion depends on active MT1-MMP recycling (Monteiro, Rosse et al. 2013), whereas filopodia-driven cell invasion depends on integrin recycling.

RTKs link to integrin trafficking

RTK signaling has also been demonstrated to positively regulate integrin trafficking. More specifically, stimulation with PDGF resulted in the formation of circular dorsal ruffles, which were enriched in β1 and β3 integrins that had been taken up by macropinocytosis. Obviously, this increased integrin uptake creates an internal pool of integrins that can be subsequently recycled and promote the formation of new adhesions at the leading edge (Gu, Noss et al. 2011).

Tensin-4, an oncoprotein, and a known β1-integrin-binding partner provides an additional link between c-MET and β1 integrin traffic. More specifically, tensin-4 interacts with active c-MET, interferes with its endocytosis and leads to constitutive c-Met signaling. Therefore, the tensin-4–c-Met complex promotes cell survival, proliferation, tumor growth and cell migration, suggesting that the trafficking pathways of c-MET and β1 integrin are closely linked (Muharram, Sahgal et al. 2014).

It appears that $\alpha_v\beta_3$ integrin is implicated in VEGFR2 signaling. Pharmacological blockage of $\alpha_v\beta_3$ with small molecule inhibitors such as Cilengitide in endothelial cells, increased Rab4-dependent VEGFR2

recycling, protecting from its degradation in the presence of VEGF and increasing its levels on the cell membrane. Hence, this resulted in VEGF-driven endothelial cell migration, sprouting and tumor angiogenesis in vivo, which ultimately led to enhanced tumorigenesis (Reynolds, Hart et al. 2009).

It has been shown that Rab-coupling protein (RCP) associates with $\alpha_5\beta_1$ integrin and drives RCP-dependent recycling of $\alpha_5\beta_1$ to the plasma membrane and its mobilization to dynamic ruffling protrusions at the cell front. These RCP-driven changes in $\alpha_5\beta_1$ trafficking lead to the increase of cell migration both in 2D and 3D matrices. RCP-dependent $\alpha_5\beta_1$ recycling has also been shown to promote EGFR and c-Met recycling, further promoting cell scattering as well as tumor cell migration in 3D (Caswell, Chan et al. 2008, Muller, Trinidad et al. 2013). Therefore, RCP has been suggested to function as scaffold that promotes the association of $\alpha_5\beta_1$ integrin with EGFR and c-Met RTKs and that this drives migration of tumor cells in 3D. Notably, this pro-invasive function of RCP has been attributed to p53 gain of oncogenic function mutations, providing thus an indirect link among integrin recycling, RTK internalization and mutated p53 during oncogenesis. From a clinical perspective, this consists an interesting novel finding, since EGFR and c-Met have been reported to be activated in various cancers, and combination strategies in vivo and in vitro to inhibit both receptors have been found to be more effective than inhibition of only one (Stommel, Kimmelman et al. 2007; Kawaguchi, Murakami et al. 2009; Brevet, Shimizu et al. 2011; Xu, Stabile et al. 2011). Furthermore, the elucidation of the integrin molecules and RTKs that are activated by mutant p53 may highlight combinations of anti-integrin agents and RTK inhibitors that would be particularly effective in the treatment of mutant p53-expressing cancers (Muller, Caswell et al. 2009; Muller, Trinidad et al. 2013; Muller, Trinidad et al. 2014).

Rab proteins control pro-migratory integrin trafficking

Upon endocytosis, integrins are carried to early endosomes, where sorting into recycling and degradative pathways take place. Alterations during this process or mutation in specific Rab proteins or/and their effectors can affect the composition of focal adhesions, thereby influencing cell migration (Maritzen, Schachtner et al. 2015).

In invasive cancer cells migrating in 3D, Rab25 mediates the sorting of ligand-occupied, active-conformation $\alpha_5\beta_1$ integrin to late endosomes/lysosomes. Subsequently, integrins are transported and recycled to the plasma membrane through Chloride Intracellular Channel Protein 3 (CLIC3), increasing thus the invasive/metastatic behavior of cancer cells (Dozynkiewicz, Jamieson et al. 2012). Furthermore, Rab5 and Rab21 regulate integrin endocytosis from the plasma membrane to the early endosome. Direct interactions of integrins with Rab21 through its conserved membrane-proximal WKLGFFKR sequence have been found in the majority of the integrin α subunits and mediate β1 integrin endocytosis to EEA1-containing early endosomes (Hynes 2002). Notably, Ras GTPase-Activating Protein 1 (RASA1) displacement from Rab21 drives β1 integrin recycling from EEA1-containing endosomes back to the plasma membrane and is crucial for directional cell motility. In the same direction, Rab21 promotes cell adhesion and migration on collagen, implying thus a pro-migratory role for the endocytic trafficking of the collagen-binding integrins ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$) (Humphries, Byron et al. 2006; Pellinen, Arjonen et al. 2006).

Integrins and MMPs can be recycled using the same pathways. It has been shown that the increased expression of Rab5a, one of the three functionally redundant Rab5 genes, is predictive of increased local and distant relapse in estrogen receptor–positive (ER+) and lymph node–negative (N0) breast cancer patients. Interestingly, Frittoli and colleagues revealed that Rab5a mediates its biological activities through Rab4. In particular, they found that Rab5a promotes Rab4-dependent recycling of β3 integrin and membrane-type 1 matrix metalloprotease (MT1-MMP), thus promoting proteolytic/mesenchymal invasive programs in human breast cancer (Frittoli, Palamidessi et al. 2014).

1.7 MAP4K4

Foreword

The main focus of the present doctoral study is the function of the Ser/Thr Mitogen-Activated Protein Kinase Kinase Kinase Kinase (MAP4K4) in the dissemination of Medulloblastoma tumors (see the following chapter ‘’Objective & Aims’’). Therefore, to analytically describe the properties, functions of MAP4K4 as well as the signaling cascades it is implicated, a detailed review article is listed in the current chapter. The review article entitled ‘’The Ser/Thr kinase MAP4K4 controls pro-metastatic cell functions’’ analyzes the biology of MAP4K4 (orthologues, structure, identified substrates, signaling pathways involved), and outlines the expression of MAP4K4 in cancer tissues and its correlation with tumor progression and prognosis. Additionally, we portray the implication of MAP4K4 control in pro-invasive functions of cancer progression, and we eventually report the current therapeutic agents that target MAP4K4 functions and their potential as novel anti-metastatic agents.

1.7.1 Review Article

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The Ser/Thr Kinase MAP4K4 Controls Pro-Metastatic Cell Functions

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Abstract

The search for novel targeted therapies for major human conditions such as diabetes, cardiovascular diseases and cancer is a slow and costly process. Progress is often hampered by poor drug efficaciousness in the patients, low selectivity/specificity of the compounds and cellular evasion mechanism that are rather common in anti-cancer therapies. This is particularly true also for compounds inhibiting kinases, which in theory are optimal targets thanks to their druggable enzymatic activity. Novel targeting strategies are needed to reduce side effects and treatment failure caused by non-specific drug function and target resistance, respectively. An ideal compound will repress the relevant kinase effector function, while leaving kinase functions that are not disease-relevant unaltered. To achieve function-specific inhibition, the molecular mechanism of the drug target that governs the pathological process, must be identified.

The Ser/Thr kinase MAP4K4 is implicated in inflammatory and metabolic disorders and cancer progression. In this review, we describe the molecular effector functions of MAP4K4 that exert those activities and how they have been identified and characterized both in invertebrate organisms and mammals. We discuss how the modulation of the cellular cytoskeleton by MAP4K4 may be connected to pathological conditions such as aberrant angiogenesis and cancer metastasis, and we describe the molecular mechanisms that are so far known to be mechanistically involved in these processes.

Keywords: MAP4K4; Cytoskeleton; Cancer metastasis; Cancer progression

Introduction

MAP4K4 (Mitogen activated protein kinase kinase kinase kinase 4, also known as Hepatocyte progenitor kinase-like/germinal center kinase-like kinase (HGK) and Nck-interacting kinase (NIK)) is a serine/threonine protein kinase and member of the human STE20 family kinases; it was first identified as a kinase interactor of the adaptor protein Nck in mice [1] and short time later the human orthologue was cloned as well [2]. The MAP4K4 gene is located on chromosome 2 at position q11.2 and encodes from 33 exons a protein containing 1288 amino acids. Structurally, MAP4K4 contains an N-terminal kinase domain, a coiled-coil domain, a C-terminal hydrophobic leucine-rich citron homology domain (CNH) and two putative caspase cleavage sites [3,4]. An interdomain connecting the kinase and the CNH domain is involved in protein-protein interactions but is structurally and functionally only poorly understood. Alternative splicing results in five isoforms that display differences in interdomain composition, while kinase and CNH domain are 100% homologous. MAP4K4 is well conserved and orthologues are found across species. Genetic interference and gene expression analyses have implicated MAP4K4 activity in a plethora of cellular functions relevant for physiological and pathophysiological processes, including organ development, systemic inflammation, metabolic disorders and cancer.

The astounding bandwidth of these functions, which are further detailed below, indicates the interaction of MAP4K4 with a range of structurally and functionally different effectors. Surprisingly however, only a few interactors have so far been described, highlighting the still incomplete understanding we currently have of how MAP4K4 exerts its functions. Most of the so far identified MAP4K4 interactors are functionally associated with the modulation of the actin and microtubule cytoskeletons. It is thus tempting to speculate that MAP4K4 modulates cell behavior via targeting the underlying cytoskeleton dynamics. In this review, we will particularly focus on the role of MAP4K4 in cancer and cancer progression and discuss how MAP4K4-controlled cytoskeleton dynamics could initiate and sustain the complex morphodynamic processes associated with cancer initiation and progression.

Signaling Through MAP4K4

Since its discovery in the late nineties, only relatively few details on the regulation of MAP4K4 activity and its substrates could be revealed. This is all the more surprising, considering the wealth of its implications in development, metabolic disorders and cancer. Convincing evidence in different mammalian and fly cell systems revealed MAP4K4 functions closely associated with the tumor necrosis factor alpha (TNFα)-induced c-jun N-terminal kinase (JNK) signaling pathway [1,2,5,6]. TNFα-induced JNK activation by MAP4K4 involves signal transmission through the kinases TAK1, MKK4 and MKK7 [2]. Interestingly, MAP4K4 not only mediates TNF-α signaling but also promotes TNFα expression [2], while TNFα can also cause the specific increase in MAP4K4 expression via the transcription factors c-Jun and ATF2 [7,8]. On the one hand, this positive feed-back regulation of TNFα signaling through MAP4K4 is likely involved in physiological cell functions during development and for tissue homeostasis. On the

other hand, aberrant TNFα signaling through MAP4K4 was also linked to pathophysiological processes using genetic ablation and pharmacological inhibition approaches.

These processes include insulin sensitivity [8], systemic inflammation [9], pathogen-dependent oncogenic progression [10,11] and vascular inflammation and atherosclerosis [12]. In addition to the TNFα-receptor, growth factors (GF) such as PDGF or EGF activate MAP4K4 through receptor tyrosine kinases (RTKs) [2] and trigger the phosphorylation and activation of the MAP4K4 substrates sodium-proton exchanger [1] (NHE1) [13], ezrin, radixin, moesin (ERM) family proteins [14] and actin-related protein [2] (Arp2) [15]. Genetic studies in *C. elegans* identified an interaction between the worm ortholog of MAP4K4–MIG-15–and integrin/PAT3 [16].

Whether integrin receptor engagement could activate MAP4K4 signaling analogous to the TNFα receptor or the RTKs described above is currently not known. However, compelling evidence indicates that MAP4K4 decreases surface availability of active integrins [17], for example by inactivating integrins via moesin phosphorylation, which concomitantly competes with talin for integrin binding [18], or by accelerating their endosomal trafficking [19].

Still in the context of integrin signaling, MAP4K4 was found to be phosphorylated by the focal adhesion kinase Pyk2 [20], which is regulated by intracellular calcium levels and activated by G-protein-coupled receptors [21].

Although not directly activated by transforming growth factor ß/bone morphogenic protein (TGFß/BMP), MAP4K4 as well as the closely-related TNIK and MINK promote inhibitory phosphorylation of Thr312 in the TGFß/BMP effector R-Smads1/2/3/5/8 [22].

Somewhat counterintuitively, MAP4K4 along with its relatives MAP4K1-6, were recently also found to phosphorylate and activate the kinase Large Tumor Suppressor 1/2 (LATS1/2) under conditions of serum starvation [23], which inactivates Yes-associated protein 1 (YAP1) in the evolutionary conserved Hippo pathway. Hippo pathway activation and phosphorylation-dependent cytosolic retention of the YAP transcriptional regulator is tumor suppressive, as it prevents pro-proliferative and anti-apoptotic YAP signaling [24].

Together, a still incomplete picture begun to emerge, where MAP4K4 activation in membrane proximal, upstream compartments occurs in response to TNFα receptor, RTKs, and–possibly–also G-protein-coupled receptor ligation, to trigger JNK and Hippo pathway activation, sodium-proton exchange, actin polymerization and plasma membrane remodeling (Figure 1).

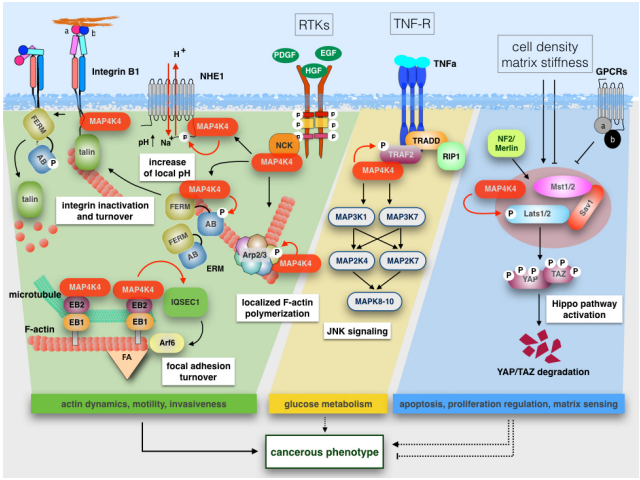

Figure 1: Overview of the major MAP4K4 signaling pathways.

From Insulin Resistance to Cell Motility: The Panoply of MAP4K4 Control of Physiological Processes

Glucose up-take and insulin function

The pathological failure of cells to respond to insulin with glucose up-take and metabolization is referred to as insulin resistance. It causes blood glucose levels to raise above the normal range, is associated with obesity, hypertension and cardiovascular diseases and eventually results in type 2 diabetes [25,26]. Causative mechanisms for insulin resistance include impaired insulin receptor (IR) signaling and the concomitantly deficient ability to restrict hepatic glucose output, as well as reduced uptake of glucose into muscle and adipose tissue through the glucose transporter GLUT4. An siRNA screen identified MAP4K4 as a negative regulator of GLUT4 control of glucose metabolism in adipocytes that impairs GLUT4 expression and function 8. Inflammation-associated TNFα regulates insulin action negatively by repressing signaling downstream of the insulin receptor and by preventing GLUT4 translocation. Depletion of MAP4K4 ameliorates systemic inflammation in a murine model by reducing TNFα expression in macrophages [9]. This led to the hypothesis that MAP4K4 could also mediate the detrimental effects in glucose metabolizing cells exposed to systemic TNFα. Indeed, silencing MAP4K4 reverted TNFα-dependent IRS2 depletion and insulin repression in beta cells [27], and it restored peroxisome proliferator-activated receptor gamma (PPARgamma) expression by de-repression of mTORC for a normalized glucose and lipid metabolism in adipocytes [28]. Consistently, microRNA 30d repression of MAP4K4 expression in pancreatic cells protected beta-cells against TNFα-induced suppression of both insulin transcription and secretion, further confirming MAP4K4 targeting as a beneficial strategy for diabetes prevention [29].

MAP4K4 has also been suggested as a potential biomarker in non-obese type 2 diabetes, since decreased MAP4K4 expression rates-attributed to increased methylation of the MAP4K4 promoter-were observed in non-obese type 2 diabetes patients compared to healthy subjects [30]. Further *in vivo* studies demonstrated that MAP4K4 deletion led to decreased fasting blood glucose concentrations and

enhanced insulin signaling in adipose tissue and liver [31], and that MAP4K4 mediates hyperinsulinemia in chronic obesity by promoting islet hypertrophy and insulin secretion from pancreatic cells [32]. MAP4K4 is also implicated in vascular inflammation and the formation of atherosclerotic plaques, where it enhances TNFα-induced vascular endothelial permeability, macrophage recruitment and adhesion [12].

These clear indications of the functional significance of MAP4K4 in inflammation-associated pathologies call for the use of therapeutics specifically targeting MAP4K4. However, the discovery of conditional MAP4K4 deficiency in T cells causing systemic inflammation and insulin resistance in mice sound a note of caution [33]. In T cells, MAP4K4 directly phosphorylates TNF receptor-associated factor 2 (TRAF2), leading to its degradation, repression of IL-6 production and the concomitant suppression of Th17 cell differentiation, which triggers an insulin resistant phenotype. Another study revealed that MAP4K4 expression in CD4+ T-cells restricts lung inflammation in response to the environmental pollutant ozone-oxidized black carbon (oBC), which is characterized by increased numbers of macrophages, lymphocytes, neutrophils, cytokine secretion and bronchoalveolar lavage fluid accumulation (BALF) [34]. Finally, mice lacking endothelial MAP4K4 displayed lymphatic defects such as dilated lymphatic capillaries, insufficient lymphatic valves, and impaired lymphatic flow [35], suggesting that beneficial effects of MAP4K4 targeting in the atherosclerotic blood vasculature may have negative impact on lymphatic drainage. Thus, careful future studies will be required to assess the cell-type specific functions of MAP4K4 and their role in metabolic and inflammatory diseases.

Development and Differentiation

The Drosophila ortholog of MAP4K4 – misshapen (MSN) – was initially identified as shape control gene in the Drosophila eye [36]. Subsequent studies implicated it upstream of Drosophila JNK to control dorsal closure during late embryogenesis [5]. In mammals, MAP4K4 plays a critical role in stimulating migration of presomitic mesodermal cells away from the Primitive Streak (PS) and in promoting the differentiation of presomitic mesoderm into somites [37]. Its absence causes embryonic lethality between days 9.5 and 10.5. MAP4K4 is also involved in the control of epithelial to mesenchymal transition (EMT) during gastrulation, where it along with the p38-interacting protein (p38IP) activates p38 to downregulated E-cadherin expression [38]. E-cadherin down-regulation contributes to the mesenchymal phenotype, which is prerequisite for cells to move away from the primitive streak. Interestingly, the above signaling cascade functions independently from the well characterized EMT-associated pathway that involves FGF and the Snail transcription factor, and suggests an alternative route requiring MAP4K4 activation of p38 [38].

TGFß/BMP signaling, which is involved in early development, axis formation and patterning [39], is negatively regulated by MAP4K4 and prevented from exerting its correct function during Drosophila imaginal disk formation [22]. Although speculative, MAP4K4 could thus be involved in spatio-temporal fine tuning of TGFß/BMP signaling during development. It is also conceivable, that alterations in this regulation in the context of aberrant MAP4K4 activation could result in developmental defects in the embryo and cancer initiation/progression in the adult organism. In addition to its role in the developing organ, MAP4K4 also contributes to reshaping the adult organism, where it restricts differentiation of myoblasts into myotubes [40] or in enteroblasts, where it controls differentiation by phosphorylation of the Hippo pathway core component LATS1/2 and concomitant degradation of the transcriptional regulators YAP/TAZ [41].

Axon Navigation and Neurite Outgrowth

Axonal migration during development is a complex process that controls the correct orientation and connection of axons. MAP4K4 is involved in axonal navigation and neuronal pathfinding with the first evidence being identified in Drosophila with the identification of Msn/MAP4K4 control of photoreceptor pathfinding [42]. Shortly after, the genetic interaction of MIG-15/MAP4K4 and the integrin Ina-1 was found to be required for proper axon navigation in *C. elegans* [16]. While MIG-15 appears to be dispensable for lamellipodia and filopodia dynamics during growth cone outgrowth and cell migration, it is required for proper directional control of neuronal outgrowth [43]. Comparative time lapse imaging of growth cone dynamics revealed that MIG-15 mechanistically interacts with the ezrin/radixin/moesin ortholog ERM-1 at the rear end of the growth cone, to restrict lateral protrusions and to maintain directional migration [44]. In mammalian cells, MAP4K4 was identified in a high-throughput screen for regulators of neurite outgrowth. In contrast to its neuro-promoting functions during development, MAP4K4 depletion in cerebellar granule neurons significantly increased the length of regenerating neurites, suggesting that MAP4K4 in mammals restricts neuronal outgrowth [45]. This finding was confirmed by an unrelated study using a militarinone-inspired 4-hydroxy-2-pyridone inhibitor targeting MAP4K4, which resulted in neuritogenesis [46]. MAP4K4 inhibition is also a reported target of kenpaullone, an inhibitor of Amyotrophic Lateral Sclerosis (ALS) [47]. Consistent with a potentially destructive function of MAP4K4 for neuronal integrity, kenpaullone improved the survival of human motor neurons derived either from mouse embryonic stem cells or from ALS-patient-induced pluripotent stem cells. Interestingly, kenpaullone efficiency was partially attributed to its inhibition of the MAP4K4-TAK1-MKK4-JNK-c-Jun cell death signaling cascade.

Together, these somewhat conflicting results from developing organisms and mature neurons corroborate the relevance of proper MAP4K4 function for neuronal development and integrity. However, future research will be necessary to reconcile the stabilizing functions of MAP4K4 during neuronal development with its destructive signaling in mature neurons. It will also be of particular interest to explore whether kenpaullone could be used to treat brain cancers with increased MAP4K4 expression such as glioblastoma [3,48] and medulloblastoma [49].

MAP4K4 is Overexpressed in Solid Tumors

A comparative analysis of healthy tissues with lines of the National Cancer Institute tumor panel reveled striking up-regulation of MAP4K4 in latter, with glioblastoma cancer cell lines displaying the highest upregulation in MAP4K4 expression levels [3]. Since then, a number of studies listed below have noted increased expression and/or altered function of MAP4K4 in tumors.

Brain tumors

Transcription analyses in glioblastoma multiforme cell lines and primary samples revealed that EGFRvIII, a constitutively activated EGFR mutant, leads to specific upregulation of a group of genes including MAP4K4 [48]. Also in a glioblastoma model, MAP4K4 was

found to interact with the focal adhesion-related kinase Pyk2 and to cooperate with it for glioblastoma cell migration [20]. MAP4K4 expression is also markedly higher in pediatric Medulloblastoma, particularly in the SHH and Group 4 subgroups, compared to healthy cerebellum tissue, and promotes tumor cell migration and invasion downstream of the hepatocyte growth factor receptor c-Met [49].

Pancreatic ductal adenocarcinoma

MAP4K4 is overexpressed in 46% of stage II Pancreatic Ductal Adenocarcinoma (PDA) primary samples, with particular overexpression detected in the neoplastic epithelium compared to the PDA stroma [50]. Furthermore, multivariate statistical analyses in PDA samples revealed the association of MAP4K4 expression with poor overall and recurrence-free survival, recommending it as a prognostic marker for patients with stage II PDA [51].

Colorectal cancer

MAP4K4 and four additional genes (LYN, SDCBP, DKK1, and MID1) consist a five-gene signature in colorectal cancer highly correlated with poor overall survival of the patients. In addition, increased MAP4K4 mRNA expression per se closely correlates with metastasis, tumor invasion and decreased overall patient survival [52].

Lung adenocarcinoma

Adenocarcinoma is the most common histological subtype of lung cancer and accounts for almost half of all lung cancers. Elevated MAP4K4 expression is closely associated with lung adenocarcinoma progression and is an independent prognostic factor predicting poor overall survival [53].

Hepatocellular carcinoma (HCC)

MAP4K4 expression is increased in Hepatocellular carcinoma (HCC) specimens compared to adjacent non-tumor liver tissues, and its upregulation correlates positively with tumor size, tumor grade and intrahepatic metastasis. Consistently, high MAP4K4 expression is an independent predictor of poor prognosis in HCC patients [54].

Gastric cancer

Gastric cancer is the second most common cause of cancer-associated mortality worldwide. In more than 2/3 of gastric cancer patient samples increased MAP4K4 expression levels were detected compared to adjacent non-tumorous regions [55].

Ovarian cancer

In contrast to all other solid tumors investigated, MAP4K4 expression levels correlate negatively with tumor load in a murine model of ovarian cancer. In this study, non-transformed ovarian epithelial cells were compared to their spontaneously transformed counterparts using genome-wide transcriptome analysis. Alterations in the gene expression profile were then correlated with the profiles of four different human ovarian cancer types [56].

microRNA regulation of MAP4K4 expression in cancer

microRNAs (miRNAs) are endogenous, small noncoding RNAs, with a length of approximately [19–24] nucleotides that negatively regulate gene expression by base pairing complementary sites on the 3'-untranslated region (3'-UTR) of target messenger RNAs [57]. Several MAP4K4-targeting miRNAs were found repressed in tumors, leading thus to increased levels of MAP4K4 expression. These include Let7a in Kaposi sarcoma, miR-194 in CRC [58] and HCC [59] and miR-622 in CRC [60].

MAP4K4 Controls Pro-Metastatic Functions in Cancer Progression

Increased expression levels of MAP4K4 at mRNA or protein levels have been correlated with tumor aggressiveness and reduced patient survival. But what are the molecular events controlled by MAP4K4 that trigger or maintain the transformed cellular phenotype and promote metastatic disease? Despite research efforts ongoing for close to 20 years, this question still remains incompletely answered. Improved gene interference strategies and the development of small compound inhibitors of MAP4K4 have simplified the identification of effector pathways and putative molecular interactors. Nevertheless, the effector molecules of MAP4K4 and how their interaction with altered MAP4K4 expression or function is coupled to the biological outcome, remains enigmatic in most instances. Key biological functions controlled by MAP4K4 are reviewed in the following section.

Tumor cell growth

Ras-transformation of NIH3T3 fibroblasts enables these cells to grow independent of adhesion to a rigid matrix (anchorage independent growth). Ablation of MAP4K4 function in such NIH3T3 fibroblasts by the overexpression of a kinase-inactive MAP4K4 mutant completely abrogated anchorage independence, suggesting that MAP4K4 contributes an essential function to anchorage-independent survival and growth [3]. Knock-down of MAP4K4 caused cell cycle arrest and apoptosis in HCCs [54] and in gastric cancer cells (GCCs) [55]. Cell cycle arrest and apoptosis after MAP4K4 knock-down was accompanied by the repression of JNK, NF kappa B, and toll-like receptor (TLR) signaling in HCC cells [54], and increased the Bax/Bcl-2 ratio and decreased Notch signaling in GCCs [55]. Whether impaired HCC and GCC growth after MAP4K4 ablation is indeed the consequence of repressed JNK, NF-kappa B and TLR signaling, or rather mediated by an alternative route in these cancer cell lines is not clear. However, since MAP4K4 ablation does not cause growth arrest and apoptosis in all cancer cell lines, the susceptibility factor in the sensitive ones remains to be determined.

Cell migration and invasion

Early studies in *Drosophila* and *C. elegans* pointed towards cell shape and migration control as hallmark functions of MAP4K4 signaling [5,36,61]. Since disturbed morphodynamics and cell migration control are intimately associated with the cancerous phenotype, studies in cancer cell lines soon confirmed the pro-migratory function of MAP4K4 [3,62]. MAP4K4 implications in migration control in the context of cell transformation was also shown in macrophages infected with the protozoan parasite Theileria annulata, where parasite-induced TNFα secretion by the host cell activates MAP4K4 in an autocrine manner, to drive migration and invasion of the host cell [11]. In an inflammatory context as well, MAP4K4 promotes Kaposi Herpes virus-infected cell migration by controlling the expression of genes responsible for motility and invasiveness such as COX-2, MMP-7 and MMP-13. In HCC, MAP4K4 depletion markedly reduced cancer cell migration both *in vitro* and *in*

vivo, reduced cell adhesion to extracellular matrix (ECM) proteins and impaired the expression of NF-κB and the Matrix Metalloproteases 2 and 9 [54,63]. In the malignant pediatric brain tumor medulloblastoma, MAP4K4 is overexpressed and mediates migration and invasion downstream of HGF and EGF [49], whereas in adult glioblastoma cells, MAP4K4 was found to interact with Pyk2, a member of the focal adhesion kinase family, and to be required for migration and invasion [20]. Molecular mechanisms linking MAP4K4 activity to pro-metastatic functions Figure 2.

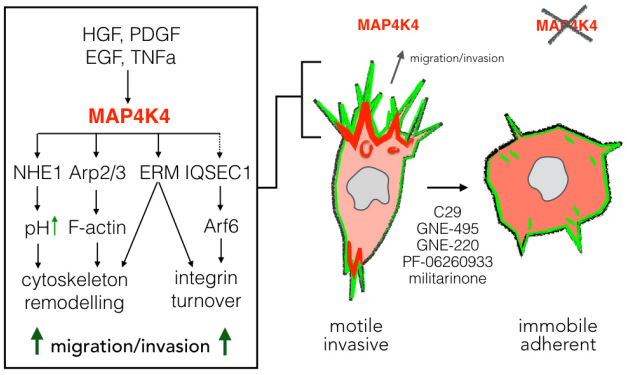

Figure 2: Upregulation of F-actin cytoskeleton turnover and membrane dynamics at the leading edge of cancer cells facilitate migration and tissue invasion. Small compound inhibitors targeting MAP4K4 block these pro-migratory functions and cells revert to a more stationary and less migratory phenotype.

Mechanistically, the identification of *C. elegans* integrin as an interaction partner of MAP4K4 during commissural axon navigation [16] hinted towards functional implication of MAP4K4 in cell adhesion and migration control. The ezrin, radixin, moesin ERM family were then identified as MAP4K4 substrates and interactors, which mediate lamellipodium extension in response to growth factor (GF) stimulation in breast adenocarcinoma cells [14] and in highly motile macrophages transformed by intracellular pathogen *Theileria annulata* [11]. Interestingly, in *C. elegans*, MIG-15 and the moesin orthologue ERM-1 function in the same pathway to direct commissural axon migration [44]. Mechanistically, MIG-15 and ERM-1 enable the stabilization of the rear end of the growth cone and the retraction of non-productive protrusions. Whether this structural stabilization of the growth cone by MIG-15 and ERM-1 in *C. elegans* is mechanistically related to lamellipodium stabilization in mammalian cells is not clear. Consistent with the notion that MAP4K4 controls cell adhesion are two recent studies describing accelerated turnover of integrin-based adhesions: On the one hand Yue et al. identified MAP4K4 as a focal adhesion (FA) disassembly factor acting in conjunction with microtubule ending binding protein 2 (EB2) and IQ motif and SEC7 domain-containing protein 1 (IQSEC1) in keratinocytes [64]. On the other hand, Vitorino et al. found that endothelial cell MAP4K4 phosphorylates moesin to displace the F-actin binding protein talin from the intracellular tail of ß1 integrin [18]. Common to both studies is that MAP4K4 activity triggers the turnover of adhesion complexes. Indeed, depletion of MAP4K4 or its inactivation causes the cells to spread and adhere more tightly [3,11,14]. Acceleration of focal adhesion turnover may thus be one function of MAP4K4 to facilitate cell displacement, in particularly also inside 3D matrix, where focal adhesions, the adhesion associated signaling complexes and their coordinated turnover controls mesenchymal cancer cell migration [65].

Cytoskeleton dynamics at the cell front during migratoion are controlled among many other factors by local pH gradients. The sodium/proton (Na+/H+) exchanger 1 (NHE1) causes spatially-restricted H+ efflux at the leading edge of cells [66]. In addition to its exchanger activity, NHE1 also acts as a membrane anchor for the cytoskeleton and an assembly point for signaling molecules [67-69]. MAP4K4 binds to and phosphorylates the C-terminus of NHE1 and causes its activation, which increases the intracellular pH in proximity of the plasma membrane [13]. NHE1 is involved in cell migration control [70-72] and associated with metastatic functions in cancer cells [73-75]. GF control of this central regulator of cortical cell functions through MAP4K4 may contribute to local remodeling of the cytoskeleton [76,77] and enable the formation of invasive membrane protrusions. Not surprisingly, targeting NHE1 has emerged as a potential strategy in tumor treatment [78].

Another possible function of MAP4K4 in cell migration and invasion control is its activity towards the actin-related protein 2 (Arp2) subunit of the Arp2/3 complex. Arp2 phosphorylation by MAP4K4 increased the actin nucleation activity of the Arp2/3 complex *in vitro* and in EGF-stimulated cells, demonstrating that MAP4K4 can couple growth factor (GF) signaling to F-actin polymerization [15]. MAP4K4 accumulates at the leading edge of cells migrating on/in either 2D or 3D matrix [3,11,14,49,79]. Thus, MAP4K4-driven F-actin polymerization at the leading edge combined with its activity towards focal adhesions make it a prime candidate for GF control of the membrane protrusions at the leading edge of cells that drive invasive cancer cell motility. Indeed, depletion of MAP4K4 in medulloblastoma cells infiltrating collagen I matrix caused a defect in protrusion formation and completely abrogated GF-induced cancer cell invasion [49].

Therapeutics Targeting MAP4K4 Functions

The broad implication of MAP4K4 in human disease conditions including metabolic and inflammatory disorders as well as cancer have incited efforts in the pharmaceutical industry to develop a specific small compound inhibitor. Genentech applied two separate fragment-based lead discovery approaches, which identified pyridopyrimidine scaffold-based compound 29 (C29) [80] and C35 [40]. Starting from a micromolar pyrrolotriazine fragment hit, Genentech developed a series of MAP4K4 inhibitors with single digit nanomolar IC50s by utilizing crystal structures and molecular modeling. Calculated properties were used to guide the optimization of solubility and *in vitro* stability, yielding compounds with excellent biochemical potencies, ligand efficiencies and favorable *in vivo* bioavailability in mice. However, as with many small compound kinase inhibitors, moderate kinase selectivity was observed and the closely related kinase Misshapen-like kinase 1 (MINK1) and TRAF2- and Nck-interacting kinase (TNIK) were also effectively inhibited by both compounds. C35 was found to be even less specific as it also blocked activities of Cdk1, Flt3, KDR, KHS1, and Mink1 by more than 75% [40]. Nevertheless, C29 was found to be a valuable tool in *in vivo* studies but its applicability suffered from the shortcoming that it was poorly tolerated when dosed repeatedly [80]. Its capability to permeate into the CSN was suspected to cause the poor tolerability. Medical chemistry efforts were therefore undertaken to reduce penetration of C29 into the CNS. The resulting GNE-495 reduced brain exposure while maintaining potent activity

and good kinase selectivity, and recapitulated the phenotypes of MAP4K4 inducible knockout mice [81]. In a search for pharmacologically active compounds targeting pathological angiogenesis, the GNE-220–yet another MAP4K4 inhibitor from Genentech–was identified. This compound prevented MAP4K4-dependent talin replacement by C-terminally phosphorylated moesin from the intracellular domain of integrin β1, and reversed membrane retraction defects in endothelial cells associated with pathological angiogenesis [18].

A virtual screening approach using the crystal structure of the MAP4K4 kinase domain was employed by Pfizer, and the compound PF-6260933 with excellent selectivity was developed [82]. However, similar to the Genentech compounds, PF-6260933 also inactivated TNIK and MINK. PF-6260933 was used in a study investigating MAP4K4 function in vascular inflammation and atherosclerosis. It phenocopied the MAP4K4 knock–down and ameliorated plaque development in a murine model for atherosclerosis [12].

MAP4K4 is highly expressed in the brain tissue and its implications in neurodegenerative disorders have been addressed in the context of a search for natural products with neurotrophic and/or neuroprotective activities [46]. One such bioactive compound - militarinone-A - was identified as metabolite of a Cordycept fungus. Militarinone-A analogs were generated and as their protein targets were not known, they were analyzed for kinase selectivity. To evaluate the neurotrophic activity of those Militarinone-A analogs, they were subjected to phenotypic screening in the human neuroblastoma cell line SH-SY5Y. Compound 11e enhanced neurite outgrowth and inhibited MAP4K4 kinase activity *in vitro*, making it a suitable starting compound for future studies related to neurological disorders and – possibly - also MAP4K4-dependent brain tumors [46].

Concluding Remarks

The amazing diversity of MAP4K4 pathological function in different human conditions calls for a more general reflection on the biological processes it is involved with, and how they are associated with a specific disease condition. In this context, the regulation of the actin cytoskeleton is a good example, as it not only enables cellular morphodynamics but also spatially controlled signaling, surface receptor trafficking and turnover and endocytic up-take, to name only a few. MAP4K4 modulates the remodeling of the F-actin cytoskeleton in response to growth factors (GF) such as HGF, EGF and PDGF or the pro-inflammatory cytokine TNFα, both by directly controlling polymerization dynamics as well as signaling mediators associated with cytoskeleton control. The tripartite mechanism control of F-actin remodeling by MAP4K4 through pH regulation [13], actin polymerization and branching [15] and F-actin anchoring [14], strongly argues for MAP4K4 effectively translating growth factor (GF) signaling into cytoskeleton alterations affecting not only the overall morphology of the cell, but also how signals are transmitted inside the cell and how surface receptors are made available. Such alterations also support the disease conditions MAP4K4 was found associated with, in particular in pathological angiogenesis and cancer metastasis. On the one hand, it will thus be of importance to further dissect the downstream effector mechanisms of MAP4K4 specific to the condition, in order to more specifically target its function in this process. On the other hand, the signaling functions of MAP4K4 should not be observed isolated, but rather in the context of its broader impact on the cytoskeleton. Consequently, inflammatory signaling triggered by TNFα and mediated by MAP4K4, should thus also be investigated under the angle of the effects MAP4K4 may have on cytoskeleton modulation. An example for such context-dependent, global modulation of cell function was observed in a host-pathogen model, where parasite-dependent, TNFα-induced MAP4K4 signaling in the host cells bifurcates into JNK expression and activation on the one hand and ERM protein-associated control of lamellipodium dynamics and invasive motility on the other hand [11]. Through its activity at different levels of cell function, specific targeting of its context-dependent actions may thus emerge as a therapy specifically tailored to the disease-causing activity, and hence reduce non-desired side-effects of treatment.

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2. Rational & Hypothesis

2.1 Rational:

Cellular and molecular mechanisms controlling migration, invasion and dissemination of Medulloblastoma (MB) are only poorly understood. The main focus of the host lab is to identify and better understand mechanisms that control the metastatic dissemination of MB. Aberrantly activated kinase signaling pathways have been linked to cancer cell migration and metastasis. One kinase with oncogenic functions is the Ser/Thr protein kinase MAP4K4, which has been linked to cytoskeletal rearrangements, cell shape and migration control, cell transformation and metastasis in human cancer (reviewed in the previous chapter). Using a host-pathogen cell transformation model, the host lab linked MAP4K4 functions to the pro-invasive phenotype of macrophages infected with the protozoan parasite *Theileria annulata*. In this study, Ma et al. had shown that parasite-induced, autocrine secretion of TNFα by the host cell activates MAP4K4, which promotes migration and invasion of the host cells. This study and studies from several other labs have thus clearly linked MAP4K4 functions to the establishment or the maintenance of a pro-metastatic phenotype, in particular through its control of cytoskeleton dynamics. However, despite the fact that MAP4K4 is overexpressed at mRNA levels in glioma, MB and MB cell lines, little was known about MAP4K4 function in metastatic brain tumors. An un-published mass spectrometry analysis of the host lab of putative MAP4K4-interacting proteins identified a number of proteins related to the regulation of the cytoskeleton and/or the endocytic machinery. Cumulatively, these observations provided a strong rational to explore MAP4K4 as pro-metastatic oncogene in MB and to determine the implication of the actin cytoskeleton and the endocytic uptake and turnover in this process.

2.2 Hypothesis:

MAP4K4 mediates MB cell migration and invasion by promoting actin dynamics and endocytic uptake.

3. Objective & Aims

3.1 Objective:

The objective of this study was to determine whether and how MAP4K4 functions control migration and invasion in MB and to gain insight in the molecular mechanism orchestrating these processes.

3.2 Aims:

1. To develop appropriate tools for the analysis of MAP4K4 function in MB
2. To determine whether MAP4K4 controls actin dynamics and endocytic up-take in MB cells
3. To determine whether the MAP4K4-controlled processes identified in aim 2 mediate pro-invasive functions in MB.

4. Results

Aim 1: To develop appropriate tools for the analysis of MAP4K4 function in MB

In this aim I explored different methods of interference with MAP4K4 expression and activity in MB cell lines. These included the downregulation of MAP4K4 expression (small interference RNA - siRNA, inducible expression of short hairpin RNA - shRNA, Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein-9 nuclease - CRISPR/CAS9 - technology) and the overexpression of MAP4K4 by ectopically expressing vectors encoding MAP4K4 wild-type (wt) or MAP4K4 kinase dead mutant (K/D).

siRNA

The cells were transfected using either Silencer Select siRNA specific for MAP4K4 or Silencer select negative control (Fig. 26A).

shRNA

Inducible shRNA DAOY cell lines were generated by lentiviral transduction pLV-H1TetO-RFP-Bsd vectors encoding either MAP4K4 shRNA or scramble shRNA. Doxycycline-containing medium was added for 48 h for shRNA induction and protein downregulation was verified by IB (Fig. 26D).

CRISPR/Cas9

MAP4K4 genomic locus was screened for the exons of interest to be targeted by sgRNAs. The exons of interest were determined based on their presence in all MAP4K4 isoforms. Subsequently, CRISPR guide RNA binding sites were screened with the ‘’Optimized CRISPR design’’ online tool (http://crispr.mit.edu/) and the candidate CRISPR sgRNAs with the minimum off-target effects (with a score of >80%) were selected. sgRNAs were designed as 20 bps sequences. The synthesized sgRNAs were cloned in BFP containing lentiviral vectors and lentiviral transduction was subsequently used to generate MAP4K4-Lenti-CRISPR MB cell lines (McComb, Aguade-Gorgorio et al. 2016).

MAP4K4 overexpression

MAP4K4-ectopically expressing MB cell lines were generated using lentiviral transduction of vectors encoding MAP4K4-wt or MAP4K4-K54R mutant (Wright, Wang et al. 2003).

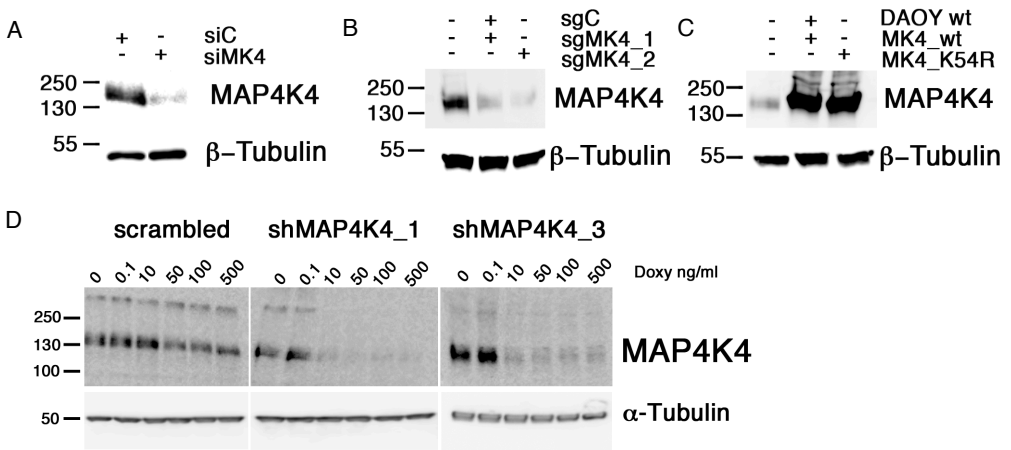

Figure 26: Methods of interfering with MAP4K4 expression in DAOY cells (Immunoblots). A, B and D: Downregulation of MAP4K4 expression using siRNA, Lenti-CRISPR constructs and shRNA respectively. C: Ectopic expression of MAP4K4 wild type (wt) and K54R kinase dead mutant.

Aim 2: To determine whether MAP4K4 controls actin dynamics and endocytic uptake in MB cells

4.1 Foreword Manuscript 1

Following the establishment of the necessary tools to study MAP4K4 function, the second aim of my project focused on the function of MAP4K4 in the regulation of MB cell motility. In particular, our studies focused on the implication of MAP4K4 in the HGF-induced cell dissemination. HGF and its receptor c-Met have been associated with poor outcome in MB patients. Furthermore, c-Met expression is increased in MB tumor samples and cell lines (Li, Lal et al. 2005), while increased expression of HGF promotes formation and growth of MB tumors in mice (Binning, Niazi et al. 2008). In parallel, increased c-Met activity has been associated with proliferation, anti-apoptosis, and migration in MB, whereas activating mutations for MB-expressed c-Met have not yet been reported (Li et al. 2005; Provencal et al. 2009; Guessous et al. 2012; Guessous et al. 2010; Kongkham et al. 2010; Onvani et al. 2012).

The molecular mechanisms and downstream effectors that mediate HGF-induced MB cell dissemination are poorly understood. Therefore, in the manuscript listed below entitled: ‘’The Ser/Thr kinase MAP4K4 drives c-Met-induced motility and invasiveness in a cell-based model of SHH medulloblastoma’’, we aimed to investigate the functional significance of the HGF-c-Met signaling pathway in MB cell dissemination. We used cell-based in vitro 2D and 3D motility assays combined with live-cell imaging and biochemical assays to examine and identify druggable mediators of HGF-c-Met-induced MB cell dissemination.

Remarkably, in the manuscript listed below, we identified MAP4K4 as a novel mediator of c-Met-induced invasive cell dissemination. We found that MAP4K4 controls of F-actin dynamics in invasive protrusions, enhancing thus MB cell invasive motility, suggesting that MAP4K4 could present a putative target for treating growth factor-induced dissemination of MB tumors. Notably, the main findings that signify MAP4K4 involvement in the HGF-c-Met signaling pathway are depicted in Figures 4B, 5 and 6 and represent my contribution to the study.

4.1.1 Manuscript 1

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The Ser/Thr kinase MAP4K4 drives c-Met-induced motility and invasiveness in a cell-based model of SHH medulloblastoma

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Abstract

Medulloblastoma (MB) comprises four molecularly and genetically distinct subgroups of embryonal brain tumors that develop in the cerebellum. MB mostly affects infants and children and is difficult to treat because of frequent dissemination of tumor cells within the leptomeningeal space. A potential promoter of cell dissemination is the c-Met proto-oncogene receptor tyrosine kinase, which is aberrantly expressed in many human tumors including MB. Database analysis showed that c-Met is highly expressed in the sonic hedgehog (SHH) subgroup and in a small subset of Group 3 and Group 4 MB tumors. Using a cell-based three-dimensional cell motility assay combined with live-cell imaging, we investigated whether the c-Met ligand HGF could drive dissemination of MB cells expressing high levels of c-Met, and determined downstream effector mechanisms of this process. We detected variable c-Met expression in different established human MB cell lines, and we found that in lines expressing high c-Met levels, HGF promoted cell dissemination and invasiveness. Specifically, HGF-induced c-Met activation enhanced the capability of the individual cells to migrate in a JNK-dependent manner. Additionally, we identified the Ser/Thr kinase MAP4K4 as a novel driver of c-Met-induced invasive cell dissemination. This increased invasive motility was due to MAP4K4 control of F-actin dynamics in structures required for migration and invasion. Thus, MAP4K4 couples growth factor signaling to actin cytoskeleton regulation in tumor cells, suggesting that MAP4K4 could present a promising novel target to be evaluated for treating growth factor-induced dissemination of MB tumors of different subgroups and of other human cancers.

Keywords: Medulloblastoma; Cancer cell dissemination; Cell motility; c-Met; MAP4K4; Actin dynamics

Background

Medulloblastoma (MB) is the most common malignant brain tumor in children and accounts for approximately 10% of all pediatric cancer deaths. MB is thought to arise from neuronal progenitor cells harboring defects in the regulation of gene expression that normally controls growth and development of the cerebellum (Roussel and Hatten 2011). MB cells can disseminate from the primary tumor in the cerebellum throughout the central nervous system and cause metastatic disease in as many as 30% of patients at diagnosis. MB comprises a diverse set of tumors (Northcott et al. 2012a) and four molecular subgroups with differential metastatic potential, named WNT (wingless), SHH (sonic hedgehog), Group 3, and Group 4 (Taylor et al. 2012), have been classified, which remain stable from primary to recurrent MB (Ramaswamy et al. 2013). Treatments that specifically target metastatic dissemination are needed to improve patient survival and reduce treatment-related morbidity.

The receptor tyrosine kinase mesenchymal epithelial transition factor (c-Met) is activated by hepatocyte growth factor/scatter factor (HGF), its only known ligand to date, which triggers phosphorylation of Tyr1230, Tyr1234, and Tyr1235 in the intracellular domain of c-Met. c-Met phosphorylation promotes the induction of various intracellular

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signaling pathways (Trusolino et al. 2010) to control cell proliferation, survival, and mobilization through the regulation of integrin function and cytoskeleton dynamics (Trusolino et al. 2010). Aberrant c-Met activation occurs in various human cancers in different organs, including the brain, and is associated with disease progression and metastatic dissemination (Sierra and Tsao 2011; Li et al. 2005; Joo et al. 2012).

c-Met is expressed in surgical MB specimens and MB cell lines and its expression and the expression of its ligand HGF is associated with significantly worse outcome in patients (Li et al. 2005). Along with SHH, increased expression of HGF promotes formation and growth of MB tumors in mice (Binning et al. 2008). An increased level of HGF was found sufficient to drive invasiveness of orthotopically xenografted DAOY MB cells (Li et al. 2005). No activating mutation has been reported for MB-expressed c-Met to date, whereas increased c-Met activity has been linked to proliferation, anti-apoptosis, and migration in MB (Li et al. 2005; Provencal et al. 2009; Guessous et al. 2012; Guessous et al. 2010; Kongkham et al. 2010; Onvani et al. 2012). c-Met was found to increase the expression of the transcription factor v-myc avian myelocytomatosis viral oncogene homolog (MYC) (Li et al. 2008), which is the hallmark of the most aggressive form of MB (Taylor et al. 2012). Pro-metastatic functions of c-Met are supported by the hyaluronan (HA) receptor CD44 and in particular by its transcript variant CD44v6, which supports c-Met-dependent signaling (Orian-Rousseau et al. 2002). Although CD44 expression has been associated with WNT and SHH signaling in MB, it's expression has not yet been analyzed in MB (Katoh and Katoh 2009; Asuthkar et al. 2012).

The molecular mechanisms and downstream effectors that mediate HGF-induced MB cell dissemination are incompletely understood. Herein we used cell-based *in vitro* two- and three-dimensional (2D/3D) motility assays combined with live-cell imaging and biochemical approaches to investigate and characterize potentially druggable mediators of HGF-c-Met-induced MB cell dissemination.

Results

c-Met and its co-receptor CD44 are highly expressed in a subset of MB tumors and patient derived cell lines

To determine the potential clinical relevance of c-Met in larger cohorts of MB, we compared the mRNA expression levels of c-Met in the Gilbertson, the Kool and the Delattre datasets available through the R2 platform for visualization and analysis of the microarray data. As control, we used nine cerebellum samples of patients aged between 23 and 50 years. We found that the median mRNA level of c-Met and its ligand HGF in MB tumors from these three different primary sample cohorts were clearly below that of normal human cerebellum (Figure 1A). However, a sub-population of MB tumors averaging 17.5% (Figure 1A, c-Met high) showed significantly increased c-Met expression. Moreover, the same datasets revealed high mRNA expression of the c-Met co-receptor CD44 (Orian-Rousseau et al. 2002) in all MB tumor samples. By analyzing 103 primary MB tumors of the Northcott 103 dataset (Northcott et al. 2011), Onvani *et al.* described the association of c-Met with the SHH subgroup (Onvani et al. 2012). We confirmed this finding using the 285 tumors of the MAGIC dataset (Northcott et al. 2012b) (Additional file 1: Figure S1A). An analogous but less marked association was also observed for HGF (Additional file 1: Figure S1B), but not for CD44 (Additional file 1: Figure S1C). Using quantitative real-time PCR (Figure 1B) and immunoblotting (IB) approaches (Figure 1C), we detected high c-Met, CD44, and CD44v6 expression both at the mRNA and protein levels in DAOY and UW228 cell lines, and much less (c-Met) or no (CD44/CD44v6) expression in D341 and D425 cell lines. Interestingly, three bands were detected in the anti-CD44v6 blot (Figure 1C, arrowheads), suggesting the presence of different CD44 isoforms with incorporated v6 variable region. DAOY cells are sensitive to sonic hedgehog (Gotschel et al. 2013) and considered a SHH-like MB cell line, whereas D341 is considered a group 3 cell line (Snuderl et al. 2013). We confirmed surface expression of c-Met, CD44, and CD44v6 on DAOY (Figure 1D) and UW228 cell lines (not shown) by flow cytometry. This analysis revealed that >90% of DAOY cells expressed c-Met, 100% expressed CD44, while only approximately 40% expressed the CD44v6 isoform. We therefore continued our studies by focusing specifically on c-Met and by studying what effects c-Met activation by its ligand HGF may have on cell migration and invasion and which effector pathways are needed to mediate the c-Met responses.

HGF stimulation activates JNK and MAPK/ERK pathways and promotes motility

To determine dynamics of c-Met-induced ERK and JNK activation in DAOY and UW228 cells, we stimulated the cells with HGF in a time course experiment. We found that HGF stimulation of DAOY or UW228 cells promotes rapid phosphorylation of c-Met (IB p-c-Met) within five to ten min (Figure 2A) and the concomitant activation of the downstream effector extracellular-signal-regulated kinase (ERK, IB p-ERK) (Figure 2B). c-Met and ERK phosphorylations were blocked when the cells were pretreated for 2 h with the ATP-competitive c-Met inhibitor PHA-665752 (Christensen et al. 2003) but not with the non ATP-competitive inhibitor ARQ197 (Munshi et al. 2010) (Figure 2B). However, we found that 24 h ARQ197 pretreatment was necessary to block acute, HGF-induced c-Met signaling (Figure 2C). c-Met can activate the c-Jun N-terminal kinase (JNK) (Rodrigues et al. 1997), which

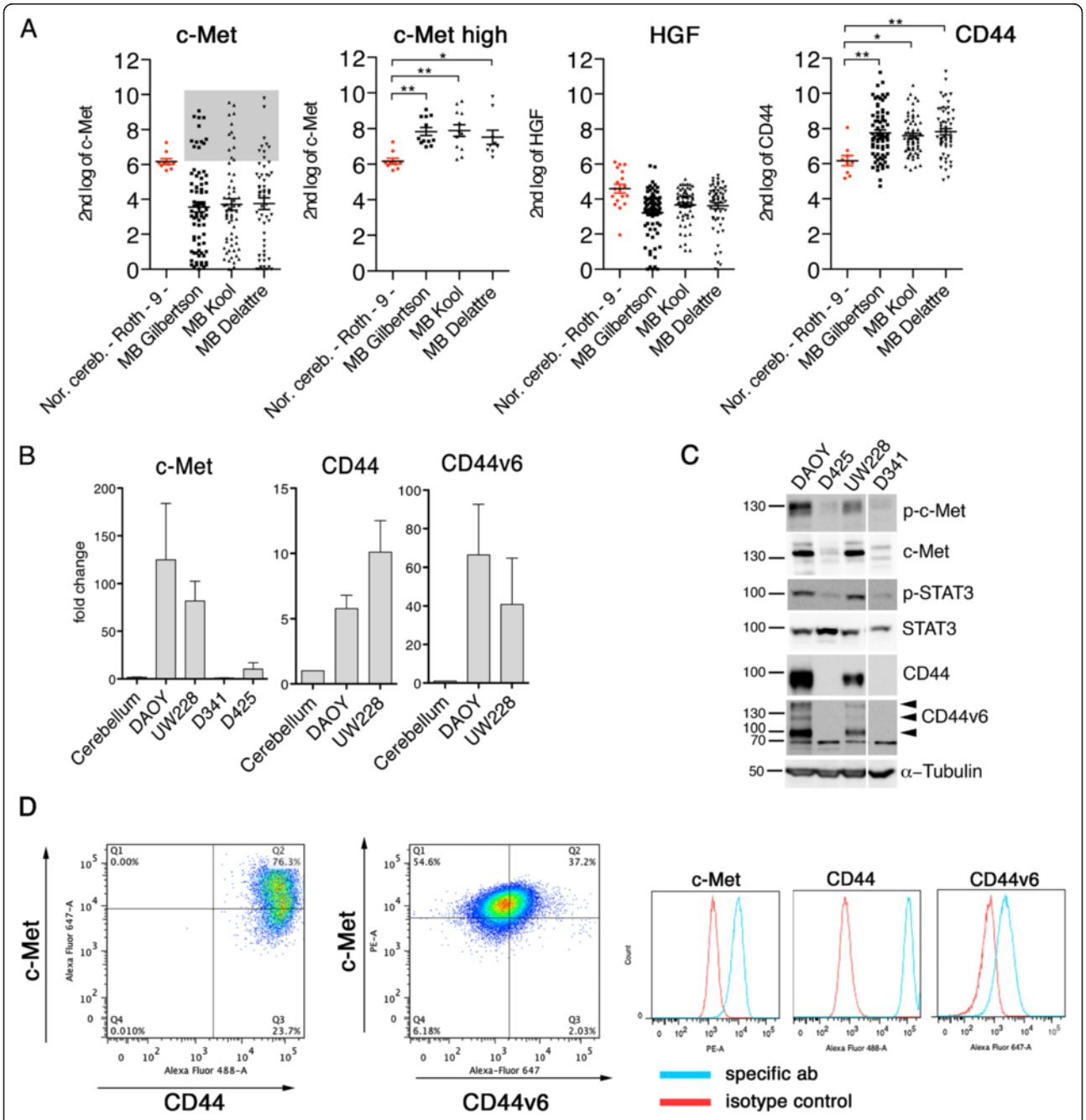

Figure 1 Expression of c-Met in medulloblastoma clinical samples and cell lines. (A) Expression analysis of c-Met, HGF and CD44 in three different MB tumor collections ($n_{total} = 195$) and in normal adult cerebellum (n = 9). **(B)** Comparative quantitative real-time PCR expression analysis of c-Met, CD44 and CD44v6 in established MB cell lines and adult cerebellum sample. **(C)** Expression and activation analysis of the c-Met pathway, CD44, and CD44v6 by immunoblotting (IB) in four different MB cell lines using the antibodies indicated to the right of the blots. **(D)** Flow cytometry analysis of DAOY cells quantifying surface expression of c-Met, CD44, and CD44v6. Dot plots compare expressions of c-Met and CD44 or c-Met and CD44v6. Histograms show relative fluorescence intensities of isotype control and specific antibody samples.

controls growth and invasion of MB cells (Zavarella et al. 2009). Consistently, we detected PHA-665752-sensitive phosphorylation of mainly the p46 isoform of JNK within five to ten minutes of HGF stimulation (Figure 2D). Interestingly, 24 h treatment with ARQ197 (Figure 2C) also caused increased JNK phosphorylation by an unknown mechanism, which was not further increase by HGF stimulation, because c-Met activity was blocked. To determine whether HGF stimulation and/or c-Met inhibition affected cell viability and/or proliferation, we performed a

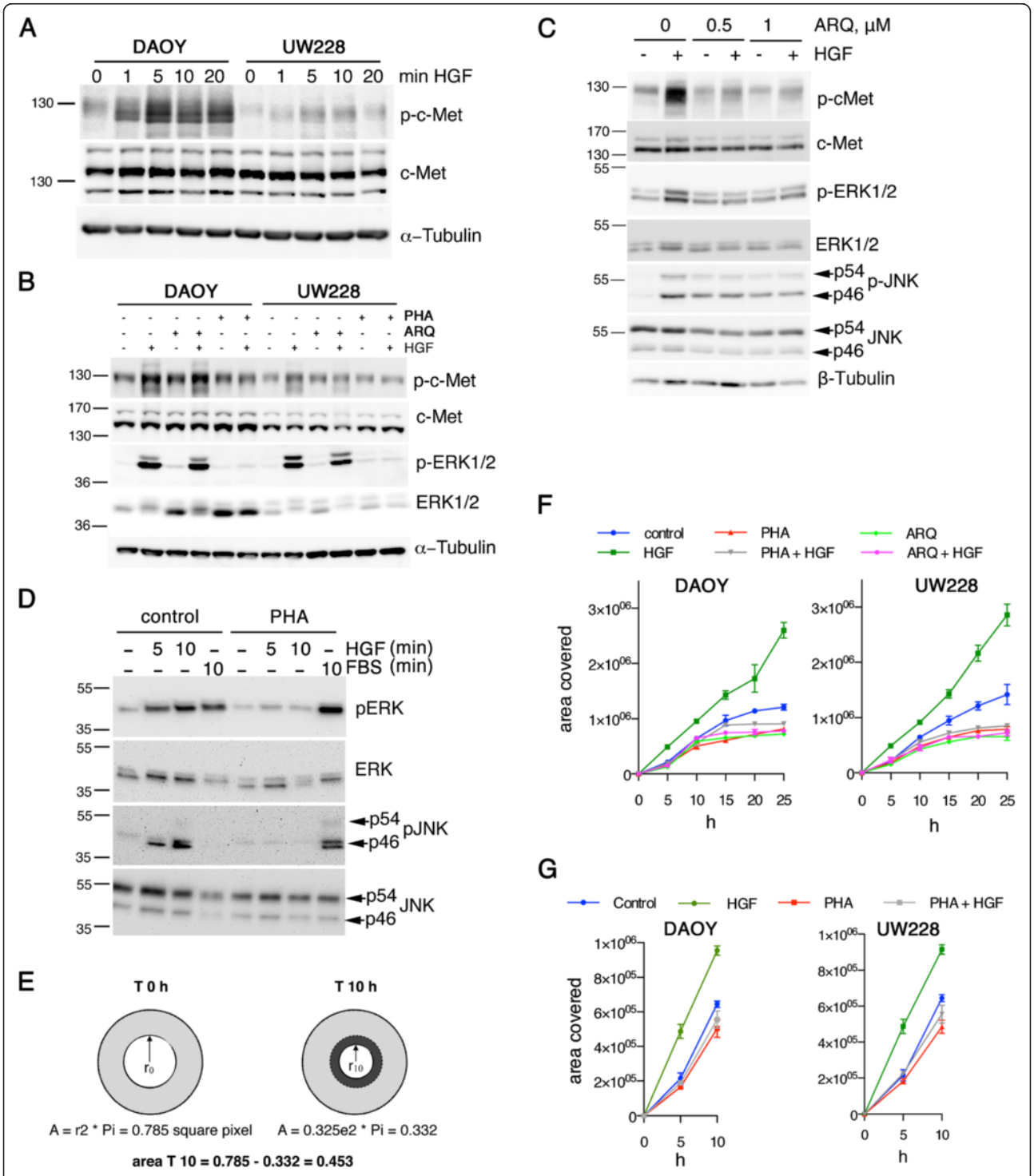

Figure 2 c-Met is activated by HGF in medulloblastoma cells and promotes wound closure. (A) IB of DAOY and UW228 lysates of cells after HGF stimulation. Antibodies used as indicated to the right of the panels. **B)** IB analysis of PHA-665752 and ARQ 197 (1 μM, 2 h pretreatment) effects on HGF-induced (50 ng/mL, 10 min) c-Met and ERK pathway activation (anti-p-c-Met and anti-pERK, respectively). **(C)** IB analysis as in B of the effect of prolonged (24 h) pretreatment with ARQ197 inhibitor on HGF-induced c-Met and ERK activation in UW228 cells. **(D)** IB analysis of HGF-induced activation of JNK and ERK in DAOY cells (20 ng/mL HGF –/+ 250 nM PHA-665752). **(E)** Schematic showing how the area covered by the cells in the Oris migration assay was determined. Light grey: cell monolayer at T_{0h}. White: gap after removal of the plug. Dark grey: area covered by cells at T_{10h}. **(F)** Quantification (means + S.D.) of Oris migration assays using DAOY or UW228 cells in HGF-treated (20 ng/mL) medium containing 0% FCS and pharmacological inhibitors (PHA-665752 and ARQ 197, both at 125 nM). **(G)** As F) but progression of gap closure shown for 0–10 h only.

tetrazolium salt WST assay on DAOY and UW228 cells treated with various combinations of HGF and PHA-665752 or ARQ197. Corresponding to c-Met expression levels (high in DAOY and UW228, low in D425), proliferation/viability was effectively reduced by the c-Met inhibitors in DAOY and UW228 cells and only moderately affected in D425 cells (Additional file 2: Figure S2). To monitor HGF-induced cell migration, we used the Oris migration assay (Gough et al. 2011) (Figure 2E) and measured the effect of HGF-c-Met signaling on the cells' capability to close a circular gap created by the insertion of a rubber stopper into the well that prevented cell attachment and growth (Figure 2F). Using time lapsed video microcopy imaging, we found that HGF treatment significantly accelerated gap closure within 24 h - both under serum-free (Figure 2F) and 10% serum (Additional file 3: Figure S3A) conditions. Importantly, time-lapse imaging showed that HGF treatment strikingly increased migration already within 5 h of incubation (Figure 2G). In DAOY cells, PHA-665752 treatment in the absence of ectopically added HGF reduced gap closure by nearly 50%, suggesting that an endogenous or a serum-derived factor activates the c-Met signaling axis and promotes pro-migratory signals (Additional file 3: Figure S3B). Overall, we showed that c-Met signaling was active in MB cells, that it was further activated by the exogenous addition of HGF and that it contributed to cell migration on 2D surfaces.

HGF promotes single cell motility and invasiveness

In assays that measure the area covered by cells such as wound healing assays or end-point Oris migration assay, it is not possible to discriminate between individual cell migration and proliferation. To determine whether HGF-induced c-Met activation indeed caused increased cell motility, we determined the speed of single cells. Towards that end, we measured the pathlength of single cells that migrated over a given time (speed) by time-lapse video microscopy. We found that HGF promoted a twofold increase in cell speed both in DAOY and UW228 cells (Figure 3A), which was blunted when c-Met was pharmacologically inhibited by either PHA-665752 or ARQ197. HGF also significantly increased single cell motility in the matrigel invasion in a c-Met-dependent manner (Figure 3B). However, the matrigel invasion assays does not permit monitoring the behavior of single cells inside a 3D matrix and measuring their speed of migration. To solve that problem, we developed a versatile micro bead invasion assay for MB cells and assessed cell dissemination from the beads into the surrounding collagen. Importantly, cells migrating inside the matrix are fully accessible for fixed- and live-cell microscopy (Figure 3C, upper). We found that HGF or epidermal growth factor (EGF) treatment promoted massive cell dissemination (Figure 3C and D). As expected, PHA-665752 prevented HGF- but not EGF-induced dissemination, confirming the specificity of this compound for the c-Met receptor tyrosine kinase. We observed that cells migrating in the collagen matrix displayed marked, F-actin rich invasive protrusions at the leading edges (Figure 3E), suggesting that local F-actin polymerization in the lamellipodia of cells is instrumental for motility. Taken together, these data demonstrate that HGF triggers dissemination of MB cells in 2D and 3D environments by accelerating motility at the single cell level. We furthermore detected enhanced local F-actin polymerization, suggesting F-actin turnover acting at the leading edge in HGF-stimulated cells as driving force.

JNK and MAP4K4 are downstream effectors of HGF-induced motility

JNK is highly expressed in the brain and controls neuronal cell migration during development (Zdrojewska and Coffey 2014) and in MB cells, HGF stimulation promoted JNK activation (Figure 2D). To test whether JNK activity was necessary for HGF-induced motility, we treated MB cells with the JNK inhibitor SP600125 (Han et al. 2001). We found that HGF-stimulated single cell motility (speed) was markedly reduced when JNK activity was blocked (Figure 4A). Interestingly, the ablation of JNK activity in the absence of HGF significantly reduced speed of single UW228 but not DAOY cells, indicating that serum-dependent motility bypasses JNK in DAOY but not in UW228 cells (Figure 4A) and suggesting different JNK pathway regulation in these closely related cell lines. We confirmed the sensitivity of HGF-induced single cell motility to JNK inhibition with the two additional JNK inhibitors JIP-1 (153–163) and AEG 3482 (Additional file 4: Figure S4). One upstream kinase of the JNK signaling pathway is the Ser/Thr kinase mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) (Su et al. 1997). MAP4K4 mediates HGF effects on anchorage-independent growth and invasiveness (Wright et al. 2003), promotes F-actin dynamics in lamellipodia and cell motility (Baumgartner et al. 2006; Ma and Baumgartner 2014) and contributes to the progression of solid tumors in humans (Collins et al. 2006; Hao et al. 2010; Liang et al. 2008; Liu et al. 2011; Qiu et al. 2012). In human MB samples of all four subgroups, MAP4K4 is highly expressed, most significantly in the SHH and Group 4 subgroups (Additional file 1: Figure S1D). Depletion of MAP4K4 using a small interfering RNA (siRNA) approach abrogated the pro-migratory effect of HGF and also significantly reduced steady-state motility (Figure 4B). Thus, HGF-Met signaling increases speed of single migrating cells through mechanisms requiring JNK activity and MAP4K4 function, suggesting that these two kinases are essential regulators of MB cell dissemination.

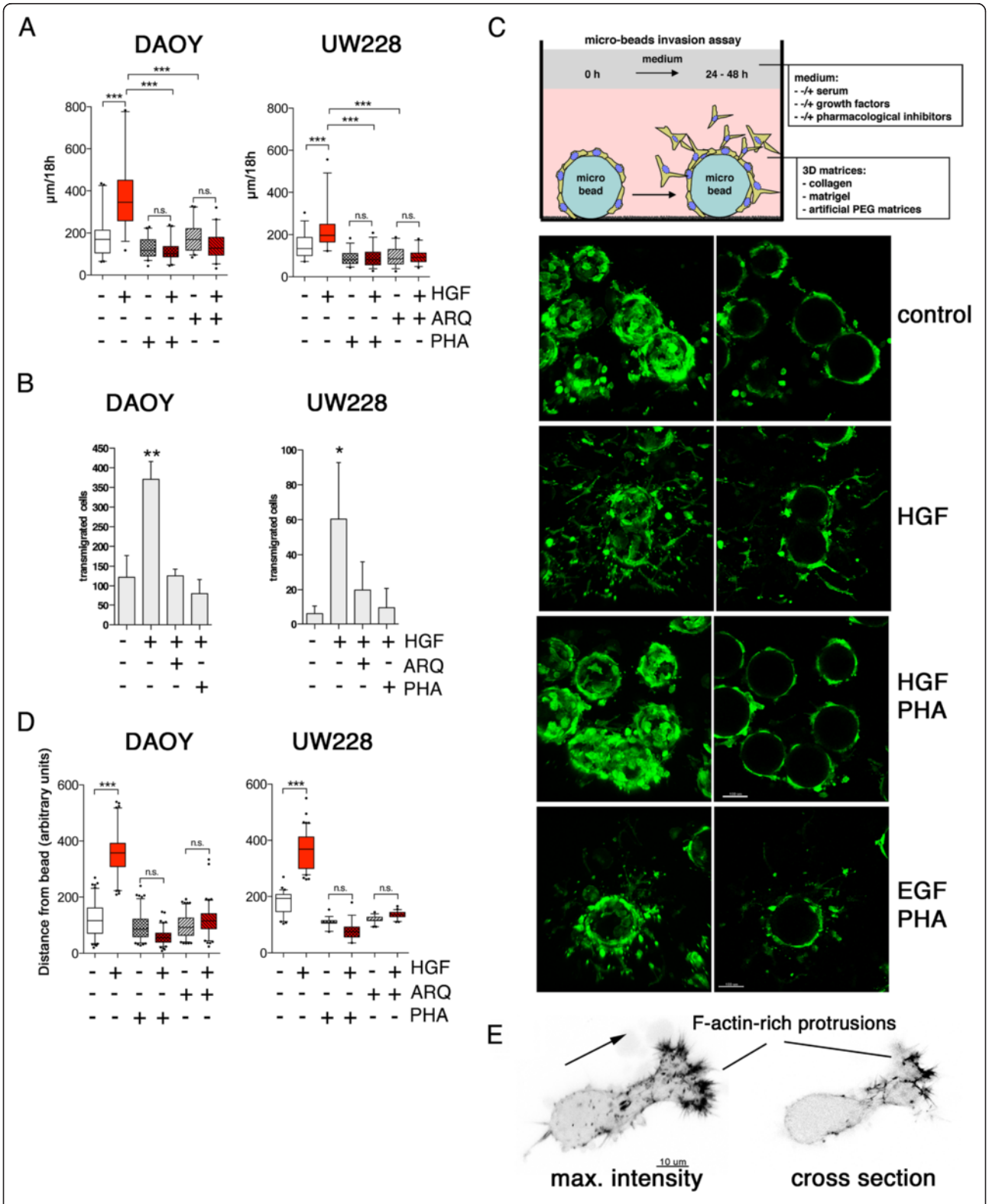

Figure 3 (See legend on next page.)

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Figure 3 HGF promotes invasive motility of single medulloblastoma cells. (A) Single cell motility of DAOY and UW228 cells was measured using live cell imaging (HGF: 20 ng/mL, ARQ197 and PHA-665752 250 nM). Box plots of three independent experiments are shown. **(B)** Boyden chamber invasion assay under conditions as described in (A). Mean total numbers of cells transmigrated and S.D. of representative triplicate experiment are shown. Statistical analysis: T-test, * = 0.0454, ** = 0.0038. **(C)** Upper: schematic of microbead invasion assay setup. Lower: microbeads coated with DAOY cells were embedded in collagen and cells were allowed to disseminate for 24 h. Confocal microscopy analysis of LA-EGFP fluorescence 24 h after embedding is shown (left: maximum intensity projection of Z-stacks, right: single cross-section through middle of beads). **D)** Quantification of mean and range of cell dissemination from microbeads shown in C (triplicate measurements, ten beads quantified per measurement, dot plot with SD). **E)** High-resolution confocal images of an HGF-induced (20 ng/mL) LA-EGFP expressing DAOY cell migrating in collagen. F-actin distribution is shown as inverted grey scale. Arrow: direction of migration. Note high F-actin content in invasive protrusions at leading edge of the cell.

HGF promotes cortical actin polymerization and membrane protrusion

Increased F-actin dynamics and cell motility indicated that c-Met could be active in lamellipodia to control F-actin dynamics in these structures. We used immunofluorescence (IF) microscopy to localize c-Met and p-c-Met in MB cells. Indeed, in lamellipodia of DAOY (Figure 5A, arrowheads) and UW228 (Additional file 5: Figure S5A) cells, we detected accumulations of c-Met and p-c-Met (Figure 5A, arrows). To test whether c-Met activation promoted cortical actin dynamics (Rottner and Stradal 2011), we stimulated MB cells with HGF and monitored immediate and late changes in cortical F-actin by immunofluorescence analysis (Figure 5B) and live cell imaging (movies Additional file 6: SM1, Additional file 7: SM2, Additional file 8:SM3), respectively. Interestingly, within 15 min we observed *de novo* synthesis of lamellipodial branched F-actin in the extension zone in HGF-stimulated cells (Figure 5B, magnifications), which was prevented when cells were pretreated with PHA-665752. We also observed accelerated and more prominent cortical F-actin turnover in HGF-stimulated UW228 cells (movies Additional file 6: SM1, Additional file 7: SM2, Additional file 8: SM3). To test whether MAP4K4 could promote cortical F-actin dynamics in MB cells, we expressed either enhanced green fluorescent protein (EGFP)-tagged wild-type (EGFP-MAP4K4-wt) or a kinase-defective (EGFP-MAP4K4-k/d) mutant of MAP4K4 in DAOY cells together with Lifeact fused to mCherry (LA-mCherry). We monitored F-actin dynamics by confocal live cell microscopy and quantified morphodynamic alterations of cell protrusions by kymography (Figure 5C and Additional file 5: Figure S5B). We found that F-actin polymerization dynamics in lamellipodia were significantly higher in cells expressing MAP4K4-wt and blunted in cells expressing MAP4K4-k/d. Interestingly, cells depleted of MAP4K4 by inducible short hairpin RNA expression (shRNA, see below) were also no longer able to respond to HGF stimulation with scattering (Figure 5D) and morphological alterations (contraction, measured as area covered per cell, 5E). Specifically, we observed that HGF-induced cell scattering evident in a culture of semi-confluent cells 24 h after HGF stimulation and resulting in dissociated cells with few cell-cell contacts, was abrogated by MAP4K4 depletion using shRNA. Reduced scattering may in part be due to reduced motility of single cells (Figure 5F). However, in shRNA MAP4K4-expressing cells, we also observed more cells with intact cell-cell contacts, suggesting that MAP4K4 effects on cell dissemination impact different levels of cell migration control. Taken together, our data show that MAP4K4 orchestrates HGF-induced morphodynamic processes and MB cell motility by controlling F-actin cytoskeleton dynamics and its depletion reduces the capability of MB cells to scatter in response to HGF.

MAP4K4 promotes HGF-induced single cell scattering and collagen invasion

To test whether MAP4K4 was driving HGF-induced invasive motility in collagen, we used MB cells expressing doxycycline (doxy)-inducible scrambled control shRNAs (shScr) or shRNAs targeting MAP4K4 (shMAP4K4) (Additional file 5: Figure S5C) in the micro bead invasion assay. Confocal microscopy imaging showed that HGF-promoted dissemination was markedly reduced in MAP4K4-depleted cells (Figure 6A). To quantify invasiveness of larger numbers of cells, we visualized cell nuclei (Figure 6B) and measured the distance between the bead and the individual nuclei (Figure 6C). We found that HGF-induced single cell dissemination in 3D was significantly reduced when MAP4K4 was depleted, both in 0% and 10% FCS medium. Importantly, HGF-stimulated shScr cells displayed considerably higher F-actin content at the leading edge than did MAP4K4-depleted cells (Figure 6D), indicating that MAP4K4-induced F-actin polymerization activity (Figure 5C–E) was also needed for forming invasive protrusions during cell migration in collagen. In conclusion, HGF promoted MB cell dissemination in collagen is driven by MAP4K4, probably by triggering the invasive, F-actin-rich membrane protrusions required for cells to invade and migrate (Figure 6E).

Discussion

In this study, we have investigated the functional significance of the HGF-c-Met signaling pathway for MB cell dissemination. We found that c-Met expression is upregulated in the SHH subgroup and in a subset of Group 3 and Group 4 MB tumors, as well as in some established

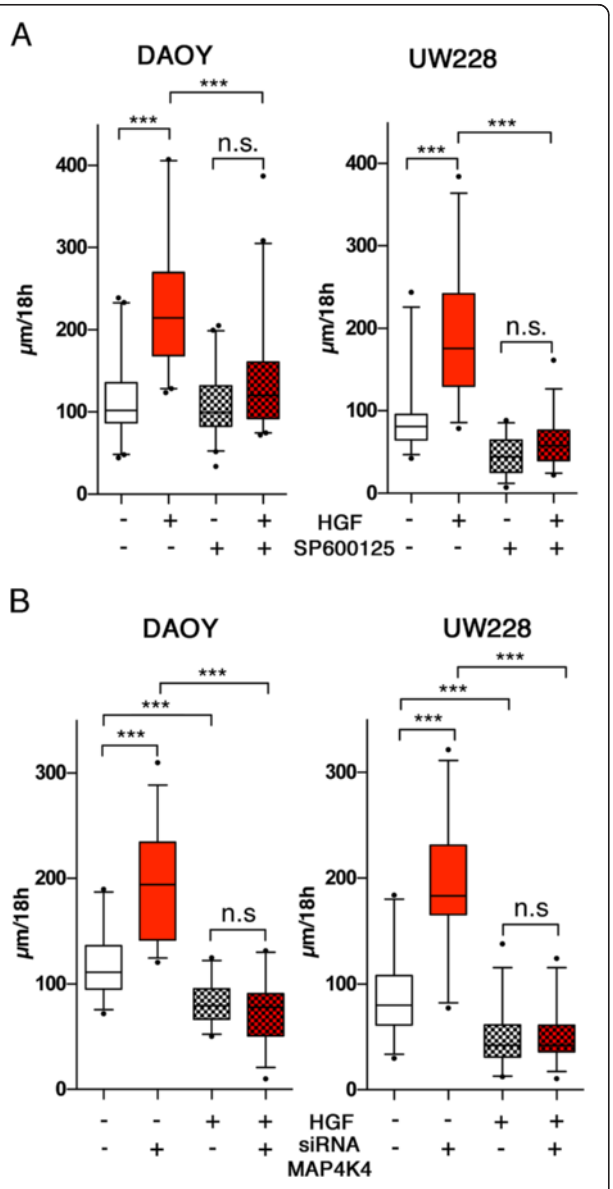

Figure 4 HGF-induced single cell motility is mediated by the Ser/Thr kinases JNK and MAP4K4. (A) Motility of single cells migrating on flat 2D surfaces in the absence or presence of HGF (20 ng/mL) and the JNK inhibitor SP665757 (10 ng/mL). Box plots of three independent experiments are shown. **(B)** Same experimental approach as in (A) but instead of using a pharmacological inhibitor, MAP4K4 was depleted from cells using validated MAP4K4-specific (+) siRNA. (–) is a negative control siRNA .

SHH MB laboratory cell lines. We demonstrated that c-Met activation by its ligand HGF promotes single cell motility of MB cells and their invasion into Matrigel and 3D collagen gels. We further showed that HGF-induced motile and invasive cell behavior requires the Ser/Thr kinase MAP4K4, which controls F-actin cytoskeleton dynamics in cellular protrusions necessary for motility and invasiveness. Thus, our studies reveal a novel, growth factor-dependent signaling circuit that promotes MB cell dissemination through MAP4K4-dependent cytoskeleton regulation, and underscore the necessity of patient stratification based on growth factor sensitivity of the tumor for rational targeting of cancer promoting signaling pathways.

Others and we have revealed a striking association of c-Met expression with SHH MB ((Onvani et al. 2012) and Additional file 1: Figure S1A) and we found that c-Met is overexpressed in approximately 18% of MB tumors compared to cerebellum controls. It is possible that c-Met could contribute to tumor progression by causing dissemination of the subset of recurrent SHH tumors reported recently (Ramaswamy et al. 2013). Importantly, c-Met function could also contribute to MB tumor cell dissemination in other subgroups by driving cell motility. However, other cellular parameters such as the capability to survive in the CSF or to colonize the new niche will be as important as well, and which could explain the discrepancy in the relative clinical outcomes between c-Met-high SHH and for example c-Met-low Group 3 tumors. Future studies examining large cohorts of patients in a subgroup-specific manner will now be required to fully appreciate the role of c-Met signaling in this context. Although the expression of the c-Met co-receptor CD44 was high in all MB tumor samples analyzed, its role in MB is unclear and further studies will also be needed here to reveal c-Met-related and un-related effects of CD44 in MB pathogenesis. Unlike CD44 expression in tumor samples, CD44 expression in MB cell lines was restricted to those expressing c-Met. Of these, only 40% co-expressed also the HGF-c-Met-interacting variant isoform CD44v6. In glioblastoma, CD44 expression conferred growth advantages and therapeutic resistance (Xu et al. 2010) and it remains to be resolved whether analogous mechanisms are also active in MB, in particular in the context of c-Met interaction with CD44v6.

Several earlier studies have implicated a role of HGF-c-Met in MB growth and dissemination and scratch wound healing assays revealed the involvement of c-Met in wound closure (Kongkham et al. 2010). However, it remained unclear whether c-Met inhibition reduced MB cell dissemination because it impaired proliferation or because it impaired cell motility. We clarified this point by providing direct evidence that HGF-c-Met function promotes the capability of MB cells to migrate, which ultimately accelerates their dissemination both in 2D and 3D environments. It can be assumed that the dual function of c-Met, stimulation of proliferation and of single cell motility is effective in other cell types or tumor cells expressing high c-Met and explains in part the effective tumorigenic activity of this receptor.

How c-Met-induced JNK promotes MB cell motility is not known; it is possible that JNK is relevant in MB cells for proper function of the microtubule skeleton during

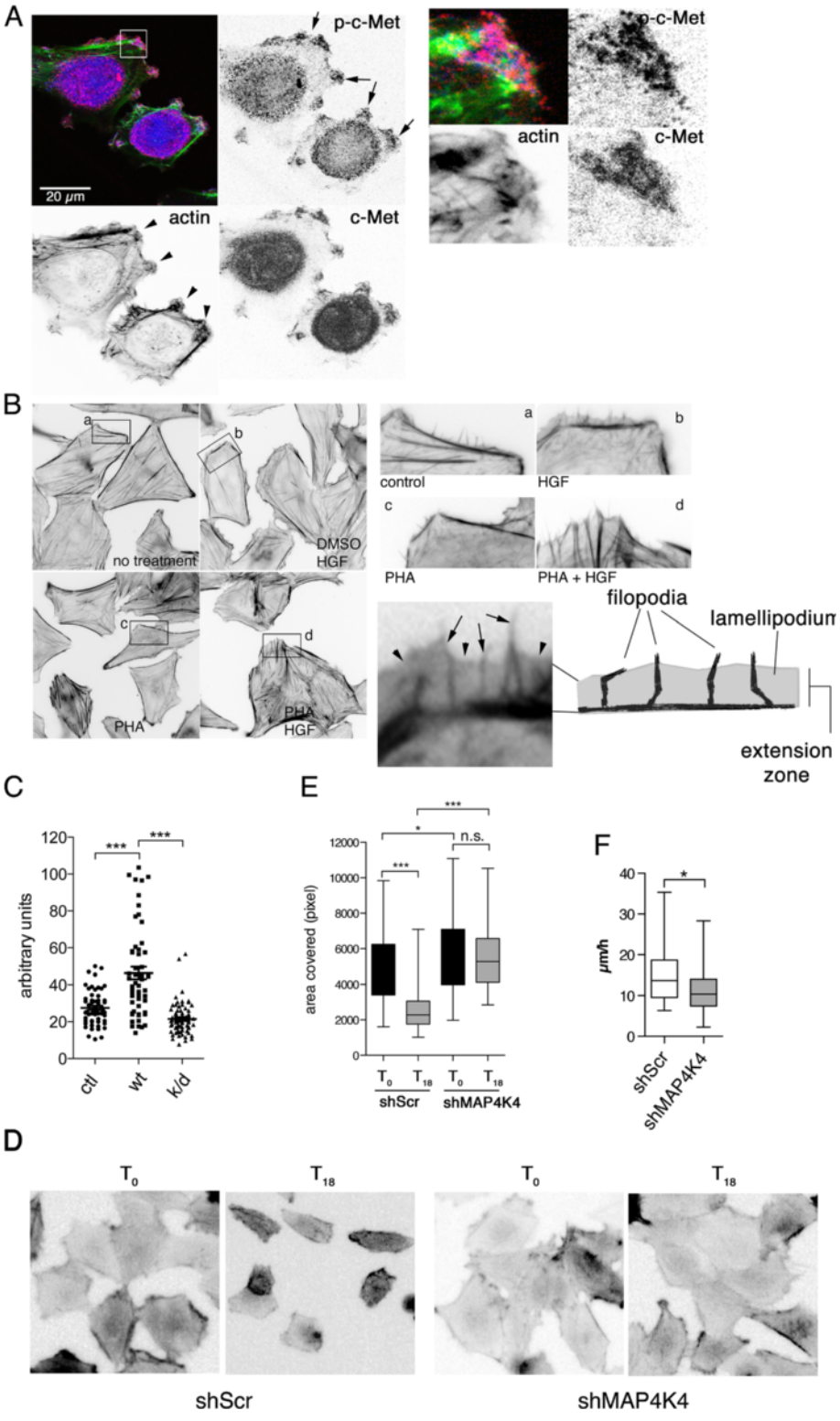

Figure 5 (See legend on next page.)

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Figure 5 HGF promotes cortical actin dynamics in medulloblastoma cells. (A) Immunofluorescence analysis (IFA) of c-Met and phosphorylated c-Met (p-c-Met) localization in lamellipodia of DAOY cells. Color overlay and inverted grey-scale images of p-c-Met (red), actin (green) and c-Met (blue) are shown. Magnifications are 4× of boxed area in overlay. Arrows indicate c-Met-rich lamellipodia. **(B)** IFA of Alexa-488-phalloidin-stained F-actin cytoskeletons in un-stimulated and HGF-stimulated (20 ng/mL, t = 10 min) DAOY cells, −/+ PHA-665752 (500 nM). Inverted grey-scale images of Alexa-488-phalloidin fluorescence are shown. Magnifications are 4× of boxed areas. Lower left magnification is 4× of sheet-like protrusion in b). Arrows: filopodia, arrowheads: leading edge of F-actin sheet (extension zone, see schematic). **(C)** F-actin dynamics in DAOY cells transfected with LA-mCherry and either enhanced green fluorescent protein-tagged, wild-type (wt) or kinase-defective (k/d) MAP4K4 were recorded by confocal live cell microscopy imaging. See Additional file 5: Figure S5 for still images of representative cells. Dot blots show protrusion lengths in control cells or cells expressing either EGFP-MAP4K4-wt or EGFP-MAP4K4-k/d. **(D)** Still images of time-lapse movies of DAOY-LA-EGFP-shScr or DAOY-LA-EGFP-shMAP4K4_1 cells stimulated with HGF (20 ng/mL). T_0 is 0 h and T_{18} is 18 h after HGF stimulation. Inverted grey-scale of LA-EGFP fluorescence (F-actin cytoskeleton) is shown. **(E)** Cells were treated as described in (D). Box plots of areas in pixels covered by individual cells quantified at T_0 and T_{18}. **(F)** Box plot of speeds of single sh control or shMAP4K4 cells in the presence of HGF. Statistical analysis: T-test (*: P = 0.0208).

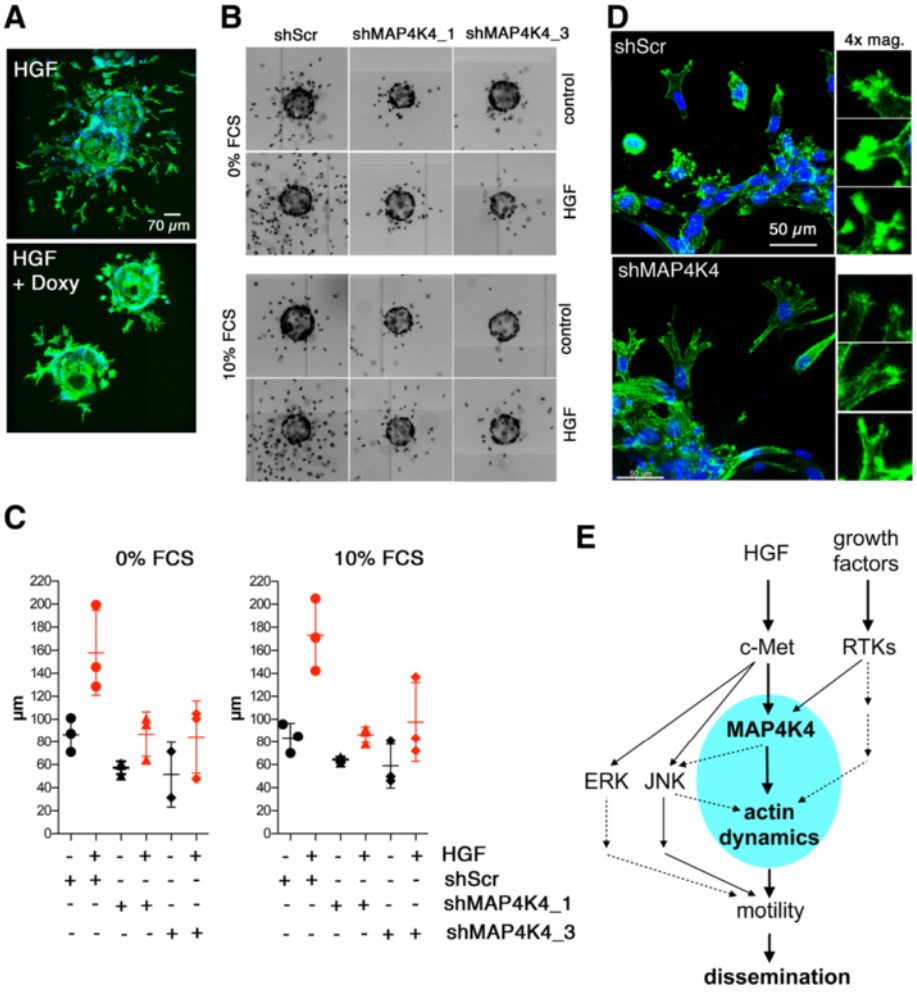

Figure 6 MAP4K4 drives HGF-induced cell dissemination in fibrillar collagen. (A) Z-stack maximum intensity projection of collagen embedded, disseminating DAOY-doxy-MAP4K4 cells. Short hairpin RNA expression was induced by doxy stimulation for 48 h before the start of the experiment (lower panel). Cells were stimulated with HGF (20 ng/mL) for 24 h. Green: F-actin, blue: DNA. **(B)** Representative montaged images of inverted grey-scale fluorescence of nuclear DNA to visualize cell dissemination. shRNA control or shMAP4K4_1/_3 expression with doxycycline and FCS and HGF treatments as indicated. **C)** Quantification of average velocities of disseminating cells with treatments as indicated. The averages of three independent experiments and S.D. are shown. **D)** High resolution confocal Z-stack of invading shScr and shMAP4K4 LA-EGFP cells. Note reduced F-actin in invasive protrusions of shMAP4K4 cells **E)** Schematic representation of signaling pathways investigated. Highlight blue is the central MAP4K4 controlled machinery that we propose to drive the dynamic remodeling of the actin cytoskeleton required for cell dissemination downstream of growth factor signaling.

motile processes through its activity toward the microtubule regulatory proteins superior cervical ganglion 10 (SCG10), doublecortin (DCX) (Zdrojewska and Coffey 2014) or microtubule-associated protein 1b (MAP1b) (Yamasaki et al. 2011). In addition to JNK, we identified MAP4K4 as a novel kinase essential for efficient dissemination of MB cells. MAP4K4 and its murine (Nck-interacting kinase), fly (misshapen) and worm (MIG15) orthologs are evolutionary conserved and control migration of both neurons (Chapman et al. 2008; Poinat et al. 2002; Shakir et al. 2006; Teuliere et al. 2011) and cancer cells (Collins et al. 2006; Wright et al. 2003; Hao et al. 2010; Liang et al. 2008; Liu et al. 2011; Qiu et al. 2012). Although its function downstream of HGF has been suggested (Wright et al. 2003), our findings are the first to demonstrate the involvement of MAP4K4 downstream of c-Met in tumor cells. Depletion of MAP4K4 reduced dissemination and the accumulation of F-actin in focal invasion structures. This finding is consistent with established functions of MAP4K4 as a regulator of cortical actin dynamics (Baumgartner et al. 2006; Poinat et al. 2002; Teuliere et al. 2011; Ma and Baumgartner 2014; Wright et al. 2003; Yan et al. 2001) and it is thus conceivable that MAP4K4 triggers and coordinates spatio-temporal actin polymerization and turnover, both of which are essential for efficient cell movement. Thus, MAP4K4 function is likely needed at the single cell level to trigger invasive cell protrusions, which in turn are necessary for motility and invasiveness of MB tumors. Although MAP4K4 is an established upstream activator of JNK (Machida et al. 2004), we could not provide convincing evidence that MAP4K4 is active in this function in MB cells as well (not shown). Hence, we concluded that while both kinases are essential for MB motility, they do probably act in parallel pathways rather than in a serial one. Considering that MAP4K4 is activated by various growth factors including HGF, PDGF (Yan et al. 2001), TNFα (Yao et al. 1999) or integrin activation (Poinat et al. 2002), we assume that several different receptor-mediated pathways trigger MAP4K4-dependent MB cell dissemination. Consequently, MAP4K4 could act as a hub to divert extracellular derived cues toward morphodynamic processes promoting motility and invasiveness (Figure 6E). Thus, we now need to further refine our understanding of upstream activators and downstream effectors of MAP4K4 in MB, because of its potential significance as a druggable anti-metastatic target for a recently synthetized novel MAP4K4 inhibitor (Crawford et al. 2014).

In summary, we have shown that the HGF-c-Met signaling pathway promotes MB cell dissemination by enabling cell dissociation, rapid movement and efficient matrix invasion of single cells. We revealed the implication of the Ser/Thr kinase MAP4K4 and its cytoskeleton modulatory functions and suggest it as a potential novel anti-metastatic target worth to investigate further. Finally, the pro-migratory functions of MAP4K4 through cytoskeleton regulation revealed herein might contribute to the metastatic progression of SHH subgroup and other MB tumors where MAP4K4 is overexpressed.

Conclusions

We have established a novel, cell-based assay to monitor cancer cell dissemination in three-dimensional matrices. We show for the first time that HGF-induced c-Met activation enhanced the speed of migration of the individual Medulloblastoma cells and show that the Ser/Thr kinase MAP4K4 is an essential mediator in this process. We conclude that MAP4K4 couples growth factor signaling to actin cytoskeleton regulation in tumor cells, suggesting that MAP4K4 could be a promising novel target to be evaluated for treating growth factor-induced dissemination of Medulloblastoma tumors of different subgroups and of other human cancers.

Methods

Ethics statement

This work was conducted according to the ethical guidelines of the University of Zürich. No donor material was used.

Expression analysis using R2 database

All data used are accessible through the open access platform R2 for visualization and analysis of the microarray data (http://r2.amc.nl). The following datasets were used: Delattre 54 MAS 5.0 – u133p2 (54 MB samples), Gilbertson 76 MAS 5.0 – u133p2 (76 pediatric MB samples, PubMed link: 22722829), Kool 62 MAS 5.0 – u133p2 (62 human MB samples, PubMed link 18769486), Northcott 103 rma_sketch – huex10t (103 primary MB samples, PubMed link 20823417) and MAGIC 285 rma-sketch – hugene11t (285 primary MB samples, PubMed link 22832581) Analysis was performed as described in (Fiaschetti et al. 2014). The nine normal cerebellum samples are from human subject aged as follows: Donor 1–25 year old male; donor 2–38 year old male; donor 3–39 year old female; donor 4–30 year old male; donor 5–35 year old male; donor 6–52 year old male; donor 7–50 year old female; donor 8–48 year old female; donor 9–53 year old female; donor 10–23 year old female.

Reagents

HGF: 0.25 μM = 20 ng/mL (Preprotech), JIP-1 (153–163) (1565, Tocris), ARQ 197 (A-1109, Active Biochemicals), PHA-665752 10 μM, AEG 3482 5 μM (Axon), (Selleck Chemicals, 10 μM). SP600125 20 μM (S5567), Doxycycline (44577) Blasticidin (15205) (Sigma-Aldrich), AEG 3482 (1291, Axon Medchem).

Cell culture

DAOY, UW228-2, D341, and D425 cells were grown as described in (Fiaschetti et al. 2014). DAOY-LA-EGFP were generated by lentiviral transduction of DAOY with cells pLenti-LA-EGFP.

Transfection

5×10^5 cells/well were seeded in 6-well plates and 24 h later transiently transfected using Jet-Pei (101–10 Polyplus), with 2.5 μg of plasmids expressing LA-mCherry (pLenti-LA-mCherry) and either MAP4K4-wt (pEGFP-C2 NIKwt) or MAP4K4-kinase dead (pEGFP-C2 NIKD152N) (Baumgartner et al. 2006).

Immunoblotting

RIPA buffer lysates were probed with the following primary and secondary antibodies: phospho-c-Met (44888, Life Technologies), c-Met (3148), phospho-STAT3 (9131), STAT3 (9132), phospho-JNK (4668), JNK (9258), phospho-ERK1/2 (9101), ERK1/2 (9102), CD44 (3578) (Cell Signaling), anti-MAP4K4 (80418, Abcam), α-tubulin (T9026, Sigma-Aldrich), and CD44v6 (MAB4073, clone VFF-18, Millipore), anti-mouse horseradish peroxidase (HRP)-linked (7076) and anti-rabbit HRP-linked (7074) (Cell Signaling). Primary antibodies were diluted 1:1000 except for α-tubulin (1:40000). Secondary antibodies were diluted 1:2000.

Immunofluorescence analysis

Cells were fixed and treated as described in (Ma and Baumgartner 2014). Primary antibodies were diluted 1:200 and incubated overnight at 4°C: α-phospho-c-Met (#44888, Life Technologies), c-Met (3148), CD44 (3578) (Cell Signaling), α-tubulin (T9026, Sigma-Aldrich), Alexa488- (A12379, Life Technologies), Cy3- (711-165-152), and Cy5-coupled (415-175-166) secondary antibodies were used (Jackson Immuno Research). Secondary antibodies and tetramethylrhodamine isothiocyanate-coupled phalloidin (Sigma-Aldrich) were diluted 1:500. Images were acquired on an Axioskop 2 mot plus fluorescence microscope (Zeiss).

Confocal live cell imaging

DAOY and UW228 cells stably expressing LA-EGFP were seeded in serum-free HEPES-buffered (25 mM) medium overnight on ibidi 8-well slides (5000 cells/well). PHA-665752 (500 nM) was added 1 h prior to and HGF (20 ng/mL) was added at the start of image acquisition in SP8 Leica confocal microscope. A 63× water immersion objective was used to acquire 60 Z-stacks of six images of EGFP fluorescence/timepoint (15 s intervals, 15 min). Average intensity projections of the stacks were assembled into QuickTime movies (10 fps, 150x speed).

Oris migration assay

The Oris™ 96-well cell migration assay kit (CMA1.101, Platypus Technologies) was used (3.5×10^4 cells seeded/well). After plug removal, cells were treated without or with HGF (20 ng/ml) and PHA-665752 or ARQ197. Cell migration was monitored for 25 h using an automated ImageXpress Micro 2 (Molecular Devices) equipped with environmental control. Images were acquired at 5 h intervals with a 10× 0.2 NA Plan Apo objective (Nikon) and Roper CoolSNAP HQ camera (Roper Scientific). Wound closure was quantified using the threshold method in the MetaXpress software (Version MX 3.1.0.93).

Matrigel invasion assay

A total of 25'000 cells were suspended in complete medium and seeded on the upper side of the Matrigel-coated membrane (BD 354480). Complete medium with or without 20 ng/ml HGF as used in the lower chamber. After 24 h, transmigrated cells were fixed with 4% PFA and stained with 0.05% crystal violet.

Single cell motility assay

Cells were seeded on 96-well glass bottom plates (In Vitro Scientific)) at 40% confluency in assay medium with or without HGF (20 ng/mL) and cell motility was acquired using the ImageXpress Micro 2 microscope. Cell speed (total path length/time) was determined by manually tracking the cells at 5 min intervals for 6–18 h using ImageJ software (National Institutes of Health, USA).

Flow cytometry

Cells were detached with Accutase (A6964, Sigma-Aldrich), fixed in 0.5% PFA for 10 min and washed in 0.5% Tween 20 (P9416, Sigma-Aldrich) and collected in flow cytometry (FC) buffer (5% FBS, 0.5% BSA, 0.1% Na-azide in PBS). 0.25×10^6 cells per sample were stained with the following primary antibodies: CD44-Alexa488 (103016, 1:50), Isotype control-Alexa488 (400625, 1:50) (BioLegend), c-Met-biotin (13–8858, 1:100), c-Met (5631, 1:100) (Cell Signaling), Isotype control-biotin (13–4301, 1:100) and CD44v6 (BMS125, 1:100) (eBioscience), and Isotype control mouse IgG1 (02–6502, Life Technologies, 1:10 – 1:50). Secondary antibodies: anti-mouse-Alexa647 (A31571, Life Technologies, 1:10000) and Streptavidin-PE (12–4317, eBioscience, 1:10000). Sequential incubations (double staining) were interrupted by three washes. Sample acquisition (10000 events) in BDFACSCanto II flow cytometer (BD Bioscience).

RNA expression analysis by qRT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Basel, Switzerland) following the manufacturer's instructions 1 μg of total RNA was used as template for reverse transcription, which was triggered by random

hexamer primers and performed by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed under conditions optimized for the ABI7900HT instrument, using Gene Expression Master Mix (Applied Biosystems). Probe-primer specific for the following genes (purchased from Applied Biosystems) were used: c-Met (Hs HS01565584_m1), HGF (Hs00300159_m1), CD44 (HS01075854_m1), CD44v6 (Hs01075854_m1). The relative gene expression was calculated for each gene of interest by using the ΔΔCT method, where cycle threshold (CT) values were normalized to the housekeeping gene 18S (Hs03003631_g1) (Applied Biosystems).

Microbead invasion assay

Approximately 500 Cytodex Microcarrier beads (Sigma Aldrich C3275) per 1.25×10^4 LA-EGFP-DAOY cells/ml were mixed in FACS tubes (BD Falcon **T7597-5 J**) and incubated at 37°C for 6 h, followed by incubation under rotation for 18 h. Non-adherent cells were removed. Cell-coated microbeads were resuspended in 2.5% bovine collagen I (5005-B, Advanced BioMatrix) in 96-well plate, after polymerization of collagen overlaid with fresh medium and treated with appropriate concentrations of c-Met inhibitors or HGF. After 24 h, cells were fixed with 4% PFA and stained with Hoechst. Images were acquired using the ImageXpress microscope. The distance between the microbead and the nuclei of the invaded cells was measured using ImageJ software. Velocity was calculated as the distance of displacement/time.

Generation of inducible cell lines

Inducible shRNA DAOY cell lines were generated by lentiviral transduction. Virus was produced in HEK293T using 4.5 μg of inducible pLV-H1TetO-RFP-Bsd vectors encoding either MAP4K4 shRNA (Biosettia, sh_NM_001242559 1–4) or scramble shRNA (Biosettia) along with lentivirus packaging plasmids pRev (1 μg), pMDL (3 μg), and pVSV (1.5 μg). Lentivirus-containing supernatants were added to recipient cells in the presence of 10 μg/ml of Polybrene (AL-118, Sigma,-Aldrich). At 48 h post-transduction, the culture medium was removed and stable cells were selected with 5 μg/ml blasticidin (15205, Sigma-Aldrich). Doxycycline-containing (Sigma, 44577) medium was added for 48 h for shRNA induction and protein downregulation was verified by IB and qRT-PCR.

RNA interference

The cells were transfected using either Silencer Select siRNA specific for MAP4K4 (ID: 18096) or Silencer select negative control #1 (ID: 4390843) (Ambion). Each siRNA was used at the final concentration of 5 nM in combination with Dharmafect 4 transfection reagent (Dharmacon), according to the manufacturer's instructions. MAP4K4 (ID: 18096) or Silencer select negative control #1 (ID: 4390843) (Ambion) were used. After 24, 48, and 72 h cells were harvested for both mRNA and protein extraction, to assess gene expression by qRT-PCR and protein content by immunoblotting.

Statistical analysis

Data are represented as the mean ± SD. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparisons test (for details please see Additional file 9: Table ST1) if not otherwise stated. P-values <0.05 were considered significant. [* < 0.05, ** < 0.01, *** < 0.001].

Additional files

Additional file 1: Figure S1. c-Met and HGF is specifically increased in the SHH subgroup of medulloblastoma. Comparison of subgroup-specific expression of **(A)** c-Met, **(B)** HGF, **(C)** CD44, and **(D)** mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) in the MAGIC (n = 285) and Northcott (n = 103) datasets. Box plots show median, mean (+), and whiskers: 5–95 percentile.

Additional file 2: Figure S2. PHA-665752 and ARQ197 block proliferation/viability of medulloblastoma cells at low molar concentrations. DAOY and UW228 cells in medium containing 0% or 10% FCS were treated with PHA-665752 or ARQ197 as indicated. Proliferation and viability of the cells were measured using the WST assay at 0 h and after 24, 48, and 72 h.

Additional file 3: Figure S3. c-Met inhibitors block basal and HGF-induced gap closure in medium containing 10% fetal calf serum (FCS). (A) Oris migration assays using DAOY or UW228 cells in 10% FCS-containing medium treated with HGF (20 ng/mL) and c-Met inhibitors PHA-665752 and ARQ 197 (125 nM). Progression of gap closure over time expressed as area in pixels covered by cells is shown. **(B)** As A) but progression of gap closure shown for 0–10 h only.

Additional file 4: Figure S4. Pharmacological JNK inhibition blocks HGF-induced motility. Speed of single cells in the absence or presence of HGF (20 ng/mL) and the JNK inhibitors AEG 3482 (5 μM) and JIP-1 (10 μM) was acquired using live cell microscopy imaging. Path lengths of individual cells after 18 h are shown (bars = means).

Additional file 5: Figure S5. (A) IFA of c-Met and p-c-Met localization in lamellipodia of UW228 cells. Color overlay and inverted grey-scale images of p-c-Met (red), F-actin (green), and c-Met (blue) are shown. Magnifications are 4× of boxed area. Arrows indicate c-Met-rich lamellipodia. **(B)** Still images of representative cells from movies. Panels to the right of each image show kymographic analysis of protrusion along lines perpendicular to the cortical F-actin. **C)** Immunoblotting analysis of stable, doxycycline-inducible DAOY shControl (scrambled) and shMAP4K4_3 and shMAP4K4_3 cell lines after 48 h doxycycline treatment using concentrations as indicated.

Additional file 6: SM1F-actin dynamics in UW228 cells expressing Lifeact (LA)-enhanced green fluorescent protein (EGFP. 15 min recording time, 10 frames per second (fps), acceleration 150x.).

Additional file 7: SM2F-actin dynamics in HGF-stimulated (20 ng/ml, 3 h) UW228 cells expressing LA-EGFP. 15 min recording time, 10 frames per second (fps), acceleration 150x.

Additional file 8: SM3F-actin dynamics in HGF-stimulated (20 ng/ml, 3 h) UW228 cells expressing LA-EGFP treated with PHA (250 nM). 15 min recording time, 10 frames per second (fps), acceleration 150x.

Additional file 9: ST1List of statistical analyses performed.

Abbreviations
2D/3D: Two- and three-dimensional; c-Met: Mesenchymal epithelial transition factor; ERK: Extracellular-signal-regulated kinase; JNK: c-Jun N-terminal kinase; HA: Hyaluronan; HGF: Hepatocyte growth factor/scatter factor; MAP4K4: Mitogen-activated protein kinase kinase kinase kinase 4; Myc: v-myc avian myelocytomatosis viral oncogene homolog; MYCN: Avian myelocytomatosis viral oncogene neuroblastoma derived homolog; SHH: Sonic hedgehog; WNT: Wingless.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
KSM, DT, MM, JG, KE, GF and MB have carried out the experimental procedures. TS and MG have helped to draft the study. MB conceived the study and wrote the manuscript. All authors read and approved the final manuscript.

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4.2 Foreword Manuscript 2

Following the completion of aim 2, we asked how MAP4K4 mediates pro-invasive functions during Medulloblastoma dissemination. Therefore we investigated the underlying molecular mechanisms. Interestingly, we had observed that cells overexpressing MAP4K4-wt form presented enhanced vesicle trafficking, with both the number and the rate of the internalized vesicles being increased compared to the cells expressing MAP4K4-KD mutant (Fig. 27). Subsequently, we performed dextran particle internalization assays in MB cells and we revealed decreased dextran uptake upon MAP4K4 inhibition (Fig. 28), further confirming the above findings. Therefore, we suspected that MAP4K4 controls vesicle trafficking events. In parallel, we were aware of the crucial regulatory function of endocytic turnover in cell migration (Hattula, Furuhjelm et al. 2006; Beaumont, Hamilton et al. 2011; Rainero, Caswell et al. 2012; Nguyen, Grimm et al. 2017). Thus, we hypothesized that MAP4K4 controls cell migration through the positive regulation of endocytic processes. The strategy we followed to test our hypothesis included functional endocytosis experiments, which determined the implication of MAP4K4 in the turnover of specific cell surface receptors.

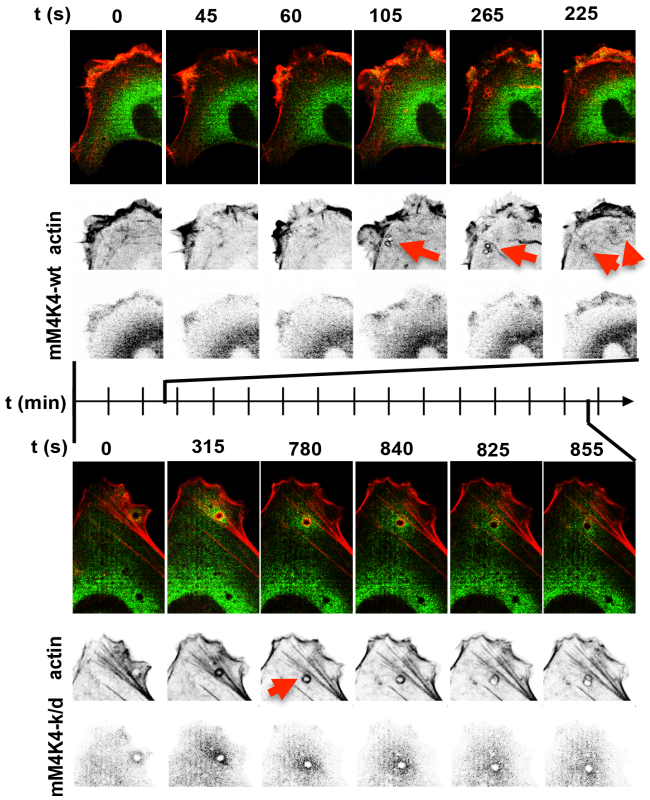

Figure 27: MAP4K4 promotes endocytic uptake. Still images of time lapse movies of DAOY cells transfected with vectors expressing MAP4K4 wild-type or mM4K4D152N (K/D) kinase dead mutant (Su, Han et al. 1997). LA-mCherry in red, EGFP-mM4K4 in green. Inverted grey scale images show actin and MAP4K4. Arrows point to the formed F-actin coated vesicles.

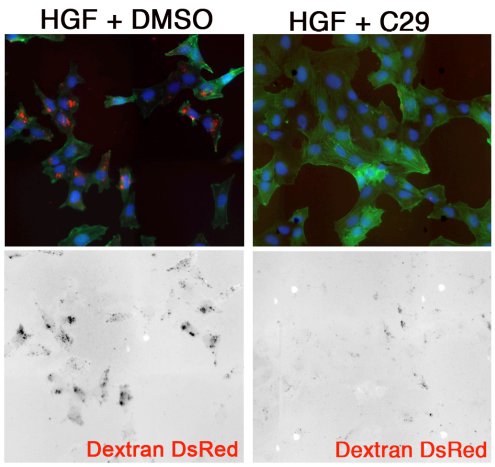

Figure 28: MAP4K4 promotes endocytic uptake. Representative images of 70 kDa DS red dextran uptake in DAOY cells treated with either DMSO, or C29. Green: F-actin, blue: DNA, Red: Dextran.

Aim 3: To determine whether the MAP4K4-controlled processes identified in aim 2 mediate pro-invasive functions in MB.

Migration and invasion of MB tumor cells can be driven and regulated by growth factors, adhesion to extracellular matrix and non-specifically induced by photon irradiation. Latter is particularly intriguing as radiation therapy represents a standard-of-care for cancer patients. In aim 3, I used dextran up-take assays, confocal microscopy and biochemical approaches in combination with pharmacological and genetic interference strategies to determine the significance of endocytic up-take and cell migration control in MB. The results of the above experiments are listed in the second publication below, which is entitled ‘’Kinase control of receptor turnover promotes tumor cell invasion in Medulloblastoma’’.

4.2.1 Manuscript 2

(Prepared for submission)

Kinase control of receptor turnover promotes tumor cell invasion in Medulloblastoma

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Abstract

Background:
Medulloblastoma (MB) cell dissemination causes metastatic disease associated with poor prognosis and high morbidity. Little is still known about intrinsic regulators of MB cell migration and invasion, the cell functions that enable metastatic dissemination in the patients. We therefore sought to identify regulators of cell migration and invasion in MB and to determine the underlying molecular mechanisms that promote the cell dissemination process.
Methods:
We used human MB tumor tissue micro array and patient-derived xenograft samples to determine protein expression levels of MAP4K4, a potentially pro-metastatic Ser/Thr kinase. We used genetically engineered MB cell lines, biochemical and confocal microscopy analysis and organotypic cerebellum slice culture to explore the consequence of altered MAP4K4 expression or activity on MB cell motility control.
Results:
We found that MAP4K4 is overexpressed in primary MB tumor samples and identified it as a driver of migration and invasion downstream of growth factor signaling and photon irradiation in MB cell lines. We found that MAP4K4 mediates pro-metastatic cell functions by enhancing reception and transmission of microenvironmental cues. We demonstrate that MAP4K4 enhances endocytic activity and accelerates activation and turnover of integrin and growth factor receptors. The consequence of these novel functions of MAP4K4 is aberrant actin based-motility and invasiveness.
Conclusions:
Our study identified a novel function of MAP4K4 as a facilitator of endocytic trafficking and receptor turnover in response to micro-environmental cues. This function enables oncogenic signaling and morphological alterations facilitating migration and invasion, which both are of high relevance for metastatic progression. These pro-metastatic activities render MAP4K4 a potential novel therapeutic target for the treatment of metastatic MB.

Key words:
Medulloblastoma, MAP4K4, cell migration and invasion, receptor trafficking

Introduction

Medulloblastoma (MB) is the most common malignant brain tumor in childhood and is a leading cause of cancer-related deaths in children. MB arises in the cerebellum, locally infiltrates and has the propensity for leptomeningeal spread through the cerebrospinal fluid (CSF). MB has been molecularly classified in several subgroups, which currently include WNT (Wingless), SHH (Sonic hedgehog) p53-wild-type and SHH TP53-mutant, as well as the two non-WNT/non-SHH groups 3 and 4 (Louis, Perry et al. 2016). Surgical resection, chemotherapy as well as local and craniospinal irradiation are the current treatment modalities for MB, and survival rates of approximately 70% can be reached (Sanders, Onar et al. 2008). However, these therapeutic strategies are associated with substantial side effects in the developing central nervous system of the patients and cause high morbidity. Moreover, metastatic and recurrent MB remain clinically very challenging diseases with high mortality rates. Therefore, therapies that target tumor cell infiltration and metastatic dissemination specifically are urgently needed to halt the progression towards recurrent or metastatic disease.

Cancer cell infiltration is a complex morphodynamic process relying on aberrant signaling, enhanced cytoskeleton dynamics as well as elevated vesicle trafficking for membrane receptor turnover and extracellular matrix (ECM) remodeling (Sanz-Moreno and Marshall 2010; Wolf and Friedl 2011; Maritzen, Schachtner et al. 2015). A key role is ascribed to the integrin adhesion receptors, which are critically involved in cell migration both under physiological and pathophysiological conditions (Huttenlocher and Horwitz 2011). Their redistribution through internalization and endocytic recycling leads to the establishment of new focal adhesions at the leading edge of the migrating cell and the formation of invasive structures such as invadopodia and filopodia (De Franceschi, Hamidi et al. 2015; Jacquemet, Hamidi et al. 2015), which both are necessary for cell migration in complex tissue environments. Thus, the aberrant recycling, activation and/or cell surface presentation of integrins can markedly enhance the invasive behaviour of cancer cells (Ramsay, Keppler et al. 2007; Ramsay, Marshall et al. 2007; Muller, Caswell et al. 2009; Mai, Veltel et al. 2011; Rainero, Caswell et al. 2012). Consequently, trafficking of ß1 integrin has been identified as novel therapeutic target for anti-metastatic therapy (Nguyen, Grimm et al. 2017). Analogously, endocytic turnover of receptor tyrosine kinases (RTKs) such as c-Met (Barrow-McGee and Kermorgant 2014), FGFR (Auciello, Cunningham et al. 2013) or EGFR (Amessou, Ebrahim et al. 2016) determines strength, specificity and duration of signaling.

One kinase involved both in integrin activation (Yue, Xie et al. 2014; Vitorino, Yeung et al. 2015) and RTK signal transmission towards migration and invasion (Baumgartner, Sillman et al. 2006; Santhana Kumar, Tripolitsioti et al. 2015) is the serine/threonine protein kinase MAP4K4 (Mitogen activated protein kinase kinase kinase kinase 4, also known as Hepatocyte progenitor kinase-like/germinal center kinase-like kinase (HGK) and Nck-interacting kinase (NIK)). MAP4K4 is a member of the human STE20 family kinases (Su, Han et al. 1997; Yao, Zhou et al. 1999) and contains an N-terminal kinase domain, a coiled-coil domain, a C-terminal hydrophobic leucine-rich citron homology domain (CNH) and two putative caspase cleavage sites (Wright, Wang et al. 2003; Delpire 2009). MAP4K4 is well conserved and orthologues are found across species (Su, Treisman et al. 1998; Poinat, De Arcangelis et al. 2002). Genetic interference and gene expression analyses have implicated MAP4K4 activity in a plethora of cellular functions relevant for physiological and pathophysiological processes, including organ development, systemic inflammation, metabolic disorders and in particular also cancer (Gao, Gao et al. 2016; Tripolitsioti, Grotzer et al. 2017).

An important step towards the development of improved, personalized therapy approaches against metastatic cancers is the identification of druggable mediators of cancer cell migration and invasion such as protein kinases, for which a number of targeting approaches have been developed (Zhang, Yang et al. 2009; Fabbro, Cowan-Jacob et al. 2012; Gross, Rahal et al. 2015). However, since pharmaceutic kinase inactivation is not always advisable, or possible, and may cause rapid emergence of resistant phenotypes, this study focused on the biology underlying the pro-metastatic kinase function. We investigated the relationship between the increased expression of MAP4K4 and the biology of migration and invasion in the pediatric brain cancer medulloblastoma. It addresses the question how MAP4K4 actin-targeting functions are coupled to migration control and specifically focuses on the regulation of receptor turnover in response to growth factor stimulation and matrix adhesion.

Materials and Methods

Reagents:

HGF: 20 ng/mL (Preprotech), Dyngo 4a (10 uM, SML0340, Sigma Aldrich), C29 (2.5 uM, 140630, Genentech) EIPA (25 uM, A3085, Sigma Aldrich), Dextran, Tetramethylrhodamine (1 mg/ml, D1818, ThermoFischer), Integrin blocking antibody (ab24693, Abcam), Normal mouse IgG (sc-2025, SantaCruz).

Cell culture

DAOY human MB cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). UW228 (Keles, Berger et al. 1995) was generously provided by John Silber (Seattle, USA). DAOY and UW228 cells were cultured as described in (Fiaschetti, Schroeder et al. 2014). DAOY LA-EGFP and UW228 LA-EGFP cells were produced by lentiviral transduction of DAOY and UW228 cells with pLenti-LA-EGFP. Cell line authentication and cross contamination testing was performed by Multiplexion by SNP profiling.

Immunohistochemistry (IHC):

IHC of FFPE samples, of the MB Tissue Microarray (TMA) and normal brain sections was performed by Sophistolab (Muttenz, Switzerland) on a Lecia BondMax instruments using Refine HRP-Kits (Leica DS9800). All buffer-solutions were purchased from Lecia Microsystems Newcastle, Ltd and used according to the manufacturer's guidelines. Paraffin-slides were de-waxed, pre-treated and incubated as follows: ER-solution 2 for 10 minutes at 95°C, ER-solution 2 for 20 minutes at 100°C and ER-solution 2 for 30 minutes at 100°C. The TMA slides were captured digitally using Axio Observer 2 mot plus fluorescence microscope (Zeiss, Munich, Germany). Anti MAP4K4 (HPA008476, Atlas) was used at a dilution of 1:50) and expression was assessed independently at 5x to 20x magnifications. The samples in the TMA slides were classified by H scores by the assessor who was blind to the clinicopathological data of the patients as high, moderate, low and negative expression.

Immunofluorescence

Cells (50,000 per condition) were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.5% Triton X-100/PBS and blocked with 10% FBS/PBS. Primary antibodies were incubated overnight and were diluted as following: mouse mc anti-MAP4K4 (MO-7, clone 4A5, Abnova 1:100), c-Met (11C4) Mouse mc (Flow Specific, 5631 Cell Signaling 1:200), phospho-c-Met (Tyr1230/Tyr1234/Tyr1235) rabbit pc (07-810, Merck, 1:150), Integrin beta 1 mouse mc (12G10) (ab30394, Abcam, 1:200), Rab7 rabbit mAb (9367, Cell signaling, 1:200). Secondary antibodies were icubated for 2h at room temperature and diluted as following: Cy3-conjugated Donkey Anti-Rabbit IgG (H+L) (711-165-152, Jackson Immunoresearch, 1:300), Cy5-conjugated Rat Anti-Mouse IgG (H+L) (415-175-166, Jackson Immunoresearch 1:300). Cells were subsequently stained with Hoechst 1:5000 (B2883, Sigma-Alrich) or phalloidin-488 (Molecular Probes, 1:500) and mounted with glycergel (Dako, C0563). Images were acquired on an Axioskop 2 mot plus fluorescence microscope (Zeiss) or in an SP8 Leica confocal microscope.

Transfection

5×10^5 DAOY-LA-EGFP cells/well were seeded in 6-well plates and 24 h later transiently transfected using Jet-Pei (101-10 Polyplus), with 2.5 µg of plasmids expressing LA-mCherry (pLenti-LA-mCherry) and either MAP4K4-wt (pEGFP-C2 NIKwt) or MAP4K4-kinase dead (pEGFP-C2 NIKD152N) (Baumgartner, Sillman et al. 2006).

Live cell imaging

DAOY cells were transfected with LA-mCherry and either pEGFP-C2 NIKwt or pEGFP-C2 NIKD152N. 24 h after transfection, cells were seeded in fresh cell culture medium in ibidi 8-well slides (5000 cells/well). mCherry and EGFP emission spectra were acquired 48 h after transfection under temperature and CO_2-controlled conditions in a SP8 Leica confocal microscope equipped with a 63× multi-immersion objective. 60 Z-stacks of six images each were acquired (0.34 μm Z-distance, 15 s intervals, 15 min). Average intensity projections of the stacks were assembled into QuickTime movies (10 fps, 150x speed). Alternatively, F-actin dynamics in DAOY cells stable expressing LA-EGFP were recorded in ibid slides as described above with a

temperature- and CO_2 controlled Zeiss Axio Observer epifluorescence microscope using a glycerol 63x objective (30 s intervals, 60 min).

Single cell motility assay

Cells were seeded in 96-well glass bottom plates (In Vitro Scientific) at approximately 40% confluency in cell culture medium without FCS for 18 h. Starvation medium was replaced with fresh pre-warmed starvation medium without or with HGF (20ng/mL). Cell motility was acquired using a temperature- and CO_2-controlled ImageXpress Micro 2 microscope for 18h. Cell speed (total path length/time) was determined by manually tracking the cells at 5 min intervals for 6 - 18 h using ImageJ software (National Institutes of Health, USA).

RNA interference:

The cells were transfected using either Silencer Select siRNA specific for MAP4K1 (ID: 22080), MAP4K2 (ID:S1688), MAP4K3 (ID:S552), MAP4K4 (ID: 18095), MAP4K5 (ID:22078), MAP3K1 (ID: S8668), MAP3K6 (ID: S17289) or Silencer select negative control #1 (ID: 4390843) (Ambion). Each siRNA was used at the final concentration of 5 nM in combination with Dharmafect 4 transfection reagent (Dharmacon), according to the manufacturer's instructions. 30 h after transfection, the cells were re-seeded for single cell migration.

Statistical Analysis

Mean ± SD are shown. Unpaired student's t-test was used to test significance of differences between two samples acquired in three independent experiments. For all other analyses one-way ANOVA repeated measures test using Bonferroni's Multiple Comparison using Prism software was performed. P-Values < 0.05 were considered significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Where indicated, asterisks show statistical significances between control and test sample.

MAP4K4 depletion by LentiCRISPR:

BFP tagged LentiCRISPR plasmids were generously provided by Dr. Scott McComb (Ottawa, Canada). Cloning of sgRNA into the LentiCRISPR plasmids was performed with a single-tube restriction and ligation method as described in (McComb, Aguade-Gorgorio et al. 2016). Production of lentiviral vectors was performed according to the standard protocol. In brief, 293T cells were transfected with using HEPES-buffered saline solution (HeBS) and 0.5 M calcium phosphate with LentiCRISPR, pVSV, pMDL, and pRev (Kindly provided by Dr. Oliver Pertz, Bern, Switzerland) in a ratio of 4.5:1.5:3:1. The media was changed after 12 h and the virus was collected at 72 h after transfection of plasmids. Viral transductions were performed using hexadimethrine bromide (H9268, Sigma-Aldrich). sgRNA sequences were screened for MAP4K4 expression in DAOY and UW228 cells by IB. The most effective sequence (Exon 3) was chosen for further experiments. The specific sg target sequences used are listed below:

Gene	Exon	Sg target sequence
MAP4K4	Exon1	TGTGATTCACCGGGATATCAAGG
MAP4K4	Exon2	GGGCGGAGAAATACGTTCATAGG
MAP4K4	Exon3	CAGGACATGATGACCAACTCTGG

Immunoblotting (IB):

RIPA buffer lysates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane using a transfer apparatus according to the manufacturer's instructions (Bio-Rad). Membranes were probed with primary antibodies against MAP4K4 (80418, Abcam), ERK1/2 (9102, Cell Signaling), phospho-ERK1/2 (9101, Cell Signaling), FAK (610088, BD Biosciences), pFAK (44-624G, ThermoFischer), c-Met (8198s, Cell Signaling), phospho-c-Met (2270325, Merck Millipore) and tubulin (T9026, Sigma-Aldrich). Primary antibodies were diluted 1:1000 -except for tubulin (1:40000)- in 5% milk. Secondary antibodies [anti-mouse horseradish peroxidase (HRP)-linked (7076, Cell signaling) and anti-rabbit HRP-linked (7074) (Cell Signaling)] were used to detect the primary antibodies and were diluted 1:5000 in 5% milk. Chemiluminescence detection was performed using ChemiDoc Touch Gel and Western Blot imaging system (BioRad) and FujiFilm LAS 3000 (Bucher biotech) Integrated density of Immuno-reactive bands was

quantified using Adobe Photoshop CS3.

RNA expression analysis by qRT-PCR:
Total RNA was isolated using RNeasy Mini Kit (Qiagen, Basel, Switzerland) following the manufacturer's instructions. 100 ng of total RNA was used as a template for reverse transcription, which was initiated by random hexamer primers. The cDNA synthesis was carried out using High capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed under conditions optimized for the ABI7900HT instrument, using TaqMan® Gene Expression Master Mix (4369016, Applied Biosystems). Primer probes specific for the following genes (4331182, Applied Biosystems) were used: MAP4K1 (Hs00179345_m1), MAP4K2 (Hs00387564_m1), MAP4K3 (Hs00269284_m1), MAP4K4 (Hs00377415_m1), MAP4K5 (Hs00179088_m1), MAP3K1 (Hs00394890_m1), MAP3K6 (Hs01085775_m1). Cycle threshold (CT) values were normalized to housekeeping gene GAPDH (Hs02786624_g1). ΔΔCT method was used to calculate the relative gene expression of each gene of interest.

Spheroid invasion assay (SIA):
2,500 cells/100 µl per well were seeded in 96 well Corning® Spheroid microplate (CLS4520, Sigma-Aldrich) (DAOY cells) and in cell-repellent 96 well microplate (650790, Greiner Bio-one) (UW228 cells). The cells were incubated at 37°C overnight to form spheroids. 60 *µ*l of the medium were removed from each well, and remaining medium with spheroid overlaid with 2.5% bovine collagen 1. Following the polymerization of collagen, fresh medium was added to the cells and treated with growth factors / cytokines and/or with inhibitors. The cells were allowed to invade the collagen matrix for 24 h, after which they were fixed with 4% PFA and stained with Hoechst. Images were acquired on an Axio Observer 2 mot plus fluorescence microscope (Zeiss, Munich, Germany) using a 5X objective. Cell invasion is determined as the average of the distance invaded by the cells from the centre of the spheroid as determined by our cell dissemination counter software aCDc (Kumar, Pillong et al. 2015). For the beta 1 integrin blockage, cells were treated with 10 ug/ml beta 1 integrin antibody or control mouse IgG for 2 h at 37^{o}C before collagen embedment.

Replating assay
Integrin activation by ligand engagement was tested as described in (Alanko, Mai et al. 2015): Cells were serum starved for 24h, detached with HyQTase, and kept in suspension in serum-free medium for 1 h stop adhesion signaling. Dyngo4a (10 µM) or equal volumes of DMSO were added to cells 15 min before replating on collagen type I (5 µg/ml) coverslips for 15 min at +37°C. Cells were subsequently fixed with 4% PFA and proceeded for Immunofluorescence. Cell imaging took place in Leica SP8 confocal microscope.

Quantification of intracellular (endosomal), activated b1 integrin
Intracellular activated beta 1 integrin on the endosomal structures was quantified using FIJI. Briefly, TIFF format images were split into subchannels. Rab7-channel (Red) images were thresholded in order to measure the area of the endosomes. Subsequently, in the beta 1 integrin channel (Blue), the integrated density that corresponded to the respective area of the endosomes was measured. Next, the division of beta 1 integrated density divided by the endosomal area was defined as the intracellular endosoml beta 1 activated integrin.

Dextran uptake assays
DAOY cells were seeded in a 24-well plate (25,000 cells/well) on top of Immunofluorescence round cover slips in a final volume of 1 ml/well. Cells were starved overnight and the following day compound treatment followed (C29: 2.5 uM, 5 hours, EIPA: 25 uM 30 min) in serum starvation medium. Subsequently medium was removed and replaced with TMR Dextran-containing medium, with or without HGF. After incubation at 37^{o}C for 30 min, cells were washed with ice-cold PBS for 5 times. Next, cells were ficed with 4 % PFA and subsequently proceeded for Immunofluorescence. Cell imaging took place in Axio Observer 2 mot plus fluorescence microscope (Zeiss, Munich, Germany)

Quantification of Dextran uptake

Dextran uptake quantification was performed using FIJI. Briefly, TIFF file images were split into subchannels. LA-EGFP-channel (Green) images were thresholded in order to measure the area of the cells. Subsequently, in the Dextran channel (Red), the integrated density that corresponded to the respective cell area was measured. Next, the division of dextran integrated density divided by the cell area was defined as the dextran uptake index.

c-Met internalization assay

c-Met internalization assay was performed as described in (Crupi, Richardson et al. 2015). Briefly, DAOY cells (10^6) were seeded in 10 cm dishes and when reached 70% confluency were starved overnight. The following day cell medium was removed while cells were on ice and was replaced with biotin-containing medium [2.5 mg of Biotin (EZ-Link Sulfo-NHS-Biotin, 21331, ThermoFischer) / 5 ml Biotinylation Buffer 1X (1.54M NaCl, 100 mM Hepes, pH 7.6, 30 mM KCl, 10 mM MgCl 2, 1.0 mM CaCl 2, 100 mM glucose, 10X] per condition, for 45 min at 4^{o}C in order to coat the cells with biotin. Next, cells were washed with ice-cold PBS and were subsequently incubated with HGF for the necessary timepoints. Following HGF incubation, cells were washed with PBS and biotinylated membrane receptors were stripped using MeSNa (Sodium-2-mercaptoethanesulfonate, 63705, Sigma-Aldrich) in 1X MeSNa Buffer (1.0 M NaCl, 500 mM Tris–HCl, pH 8.6, 10 mM MgCl 2, 1 mM CaCl 2, 10X), 20 min at 4^{o}C, 2 times. Then, cells were placed on ice, washed with ice cold PBS, lysed with RIPA buffer (500 ul/dish) and centrifuged for 15 min full speed on ice. Next, the supernatant was subjected to BCA quantification (23227, ThermoScientific). Next, 100 ug of Whole cell lysate (WCL) samples were kept as control, and then 500-800 ug of protein was added to 22 ul magnetic Streptavidin beads (88816, Thermofischer) and was incubated overnight at 4^{o}C under rotation. The following day, beads were washed with RIPA bufer for three times, using a magnetic rack. Next, beads were treated with Loading Buffer (1X) (RotiLoad, K929.1, KarlRoth), boiled for 5 min at 95^{o}C and lysate was subjected to Immunoblot. During this procedure, the necessary control samples [Total protein, TP, as well as the MeSNa stripping control, M/0] were kept and lysed along with the experimental samples.

c-Met antibody feeding assay

DAOY LA-EGFP cells (30,000) were seeded in 24-well plate Poly-L-Lysine (PLL, P9155 Sigma Aldrich) –coated coverslips in serum starvation medium. The following day cells were incubated with c-Met antibody (11C4, 56315 Cell Signaling) for 1 h at 4^{o}C. Subsequently, cells were washed three times with ice cold PBS and were then incubated with or without HGF for 30 min at 37^{o}C. Next, cell medium was removed, cells were fixed with 4% PFA and were subsequently subjected to immunofluorescence.

c-Met activation assay

DAOY cells (400,000) were seeded on Poly-L-Lysine (PLL, P9155 Sigma Aldrich)-coated 6-well plates and were overnight incubated at 37^{o}C. The following day cells were treated with compounds and/or HGF and incubated for 30 min at 37^{o}C. Subsequently cells were lysed using RIPA buffer and were immediately subjected to Immunooblot.

Results

The Ser/Thr kinase MAP4K4 is overexpressed in Medulloblastoma

Several studies detected increased expression of MAP4K4 in various solid human cancers and suggested its contribution to tumor growth and progression (Wright, Wang et al. 2003; Badea, Herlea et al. 2008; Liang, Wang et al. 2008; Hao, Chen et al. 2010; Qiu, Qian et al. 2012). To determine whether increased MAP4K4 expression could also be associated with MB, we analyzed the genomic information available for a number of MB clinical samples in the R2 genomic analysis and visualization platform. This analysis revealed increased expression of MAP4K4 in MB compared to normal cerebellum (cb) or normal brain (Fig. 1 A). We confirmed increased MAP4K4 expression at mRNA levels by quantitative real time PCR (qrt-PCR) using a small cohort of MB confirmed clinical samples (Fig. 1B). Immunohistochemistry (IHC) detected strongly increased MAP4K4 protein expression in human non-WNT/SHH MB, a moderate increase in group 3 and SHH patient-derived xenograft (PDX) and again very high expression levels in a Ptch/P53 null genetic mouse model (Fig. 1C). Consistent with the relatively low mRNA expression levels in normal cerebellum, we only detected a weak MAP4K4 signal in healthy cb tissue adjacent to the tumor (Fig. 1C,a). To explore the potential clinical significance of MAP4K4 in MB patients, we probed an MB tissue micro array (TMA) containing 68 human tumor samples and 7 cb controls. MAP4K4 expression levels were scored by H score by a pathologist into negative, low, moderate and high. This analysis revealed a clearly increased expression of MAP4K4 in tumor tissue compared to adjacent cb, with some tumors showing very high levels of MAP4K4. These data demonstrate that the expression of MAP4K4 in MB is increased, both at the mRNA and the protein level.

MAP4K4 expression in SHH MB cell model is required for migration and invasion.

A number of MB laboratory and patient-derived xenograft (PDX) lines derived from SHH or G3 MB are available. We compared MAP4K4 expression in some of those lines by qrt-PCR (Fig. 2A) or immuno blot (IB, Fig. 2B). The highest mRNA expression levels were detected in the atypical MB PDX line MedPDX311, which also displayed high MAP4K4 protein. Nevertheless, no marked differences in MAP4K4 protein levels were noticeable between the MB cell lines. However, the presence of multiple bands of variable intensities indicate variant expression in the different lines. We detected MAP4K4 in and around the nucleus in the two SHH lines DAOY and UW228, and - similar to previous observations (Baumgartner, Sillman et al. 2006; Ma and Baumgartner 2014) – in lamellipodia (Fig. 2C). Five human MAP4Ks are listed in NCBI (MAP4K1-5). We previously showed that MAP4K4 is required for efficient migration in response to hepatocyte growth factor (HGF) stimulation (Santhana Kumar, Tripolitsioti et al. 2015). To determine whether the other MAP4Ks could be also involved in migration control, we depleted each variant with individual siRNAs. We found that depletion of MAP4K1-3 had no effect on HGF-induced single cell migration in 2D, whereas depletion of MAP4K4 and MAP4K5 completely abrogated migration (Fig. 2D). Since the siRNA targeting MAP4K5 also caused marked reduction of the MAP4K4 transcript (Fig. S1A), we consider MAP4K4 the predominant kinase in particular also as it showed the highest mRNA expression (Fig S1B). To quantify cell migration and invasion, we used the spheroid invasion assay (Kumar, Pillong et al. 2015), which measures how far cells disseminate from a tumor cell spheroid into a collagen gel (Fig. 2E, schema). Distance of dissemination is quantified from the center of the spheroid using aCDc software (Kumar, Pillong et al. 2015). We depleted MAP4K4 either using lentivirus encoding tetracyclin-inducible short hairpin RNA targeting MAP4K4 mRNA or a CRISPR/CAS9 cassette guided towards *MAP4K4*. shRNA depletion of MAP4K4 in DAOY (Fig. 2E) or UW288 (not shown) cells significantly impaired HGF- and EGF-induced migration and invasion (Fig. 2E). For each we used three different constructs and identified for both approaches two constructs that led to at least 70% downregulation. Depletion of MAP4K4 by shRNA impaired HGF-, EGF- and IGF-induced migration and invasion but did only moderately affect dissemination induced by bFGF, where a significant proportion of cells are refractory to the effect of MAP4K4 depletion (Fig. 2E, S2A). CRISPR/CAS9 mediated depletion of MAP4K4 confirmed the significance of MAP4K4 function for c-Met signaling-induced migration and invasion. Moreover, the pyridopyrimidine scaffold-based compound 29 (C29) MAP4K4 inhibitor (Crawford, Ndubaku et al. 2014) prevented HGF-induced collagen invasion, indicating that MAP4K4 kinase activity is required for

the pro-migratory function of MAP4K4 (Fig. 2 G). To determine whether the increased levels of MAP4K4 detected in MB tumors per se and independent of an additional stimulus could be sufficient to trigger migration and invasion, we generated DAOY cells stably expressing either wt or the kinase dead mutant of MAP4K4 (K54R (Wright, Wang et al. 2003)). The over expression of the wt but not of K54R MAP4K4 triggered collagen invasion in the absence of HGF similar to control cells in the presence of HGF (Fig. 2H), with considerably more cells migrating longer distances. Overexpression of K54R MAP4K4 in the presence of HGF did only partially reduce migration and invasion, suggesting that it only incompletely acts as a dominant negative (data not shown). Taken together, these data demonstrate that MAP4K4 plays an essential role in growth factor induced migration control in MB cells and that overexpression of MAP4K4 alone is sufficient to trigger its pro-migratory functions in the absence of additional exogenous stimulation.

MAP4K4 mediates radiation induced migration and invasion

Radiotherapy is a standard therapy in medulloblastoma and required for preventing metastasizing disease (Halberg, Wara et al. 1991; Deutsch, Thomas et al. 1996). The role of photon radiation on the motile and invasive behavior of the tumor cells remains controversial and may be cell and context dependent (Fujita, Yamada et al. 2015). However, sub lethal doses of radiation can induce pro-metastatic phenotypes and increase the migratory and invasive potential of tumor cells. This effect can either be mediated by changes in the tumor stroma (Ohuchida, Mizumoto et al. 2004; Asparuhova, Secondini et al. 2015), or by intrinsic alterations in the tumor cells (Wild-Bode, Weller et al. 2001; Qian, Mizumoto et al. 2002; Nalla, Asuthkar et al. 2010; De Bacco, Luraghi et al. 2011). To explore the potential impact of photon irradiation on the migratory behavior of MB cells, we irradiated collagen-embedded sgControl DAOY and sgControl UW228 spheroids with different radiation doses and measured collagen invasion. We found that irradiation doses of 1 and 2 Gray (Gy) increased the migratory capability of DAOY and UW228 cells, respectively (Fig. 3A). Radiation-induced migration is similar to that induced by HGF but generally more modest (fewer cells migrating shorter distances). To determine, whether radiation and HGF synergize, we combined irradiation and HGF treatment. We observed no marked additive or even synergistic effect, indicating that irradiation either induces c-Met pathway functions or the presence of a convergence effector required downstream of both cues. To test whether MAP4K4 could be such an effector, we compared coll I invasion after irradiation of sgControl and sgMAP4K4 cells. Interestingly, both in the absence or presence of HGF, the depletion of MAP4K4 abrogated coll I invasion. To establish a better understanding of the biologically relevant differences of migration, we determined the frequency distributions of the different dot plots and measured the are under the curve above and below a cut-off point of 250. MAP4K4 depletion completely abrogated migration induced by IR beyond cut-off 250 in the absence of HGF. Coll I invasion was partially rescued in the presence of HGF in sgMAP4K4 cells, but only to a level similar to sgControl without HGF. These data indicate that MAP4K4 controls a mechanism required for both migration induced by HGF and by irradiation.

MAP4K4-dependent migration is associated with increased actin dynamics and endocytic activity

To determine in which subcellular compartment MAP4K4 functions might be relevant for migration control, we overexpressed either wt or a kinase-dead mutant (mMAP4K4-D152N) of mMAP4K4 fused to EGFP in MB cells and monitored EGFP and F-actin dynamics during migration. We observed that MAP4K4 co-localized with the dynamic F-actin at the leading edge (Fig. 4A, movie 1) and also accumulated in perinuclear regions. The pronounced ruffling activity at the leading edge was accompanied by rapid trafficking of F-actin decorated vesicles towards the perinuclear regions. In cells expressing MAP4K4 k/d, ruffling was markedly slowed and speed of vesicle trafficking reduced. Interestingly, EGFP-MAP4K4-k/d associated with the F-actin-decorated vesicles, suggesting that delayed vesicle trafficking could be related to the lack of MAP4K4 kinase function. To test whether its kinase activity is required for vesicle emergence and trafficking from dynamic lamellipodia, we compared F-actin dynamics in DMSO control treated cells and cells treated with the MAP4K4 inhibitor C29 (Crawford, Ndubaku et al. 2014). C29 completely abrogated F-actin ruffling and the emergence of F-actin coated vesicles (Fig. 4B a). Exposure of the cells to 70 kDa ds dextran caused rapid up-take of the fluorescent dextran (Fig. 4Bb). This up-take is enhanced by GFs in MB cells as dextran internalization increased in response to HGF (Fig. 4C) or EGF (Data not shown) stimulation. Pre-treatment

with C29 abrogated GF-induced dextran up-take, suggesting that MAP4K4 could mediate GF-induced endocytic activity (Fig. 4D). Consistently, depletion of MAP4K4 impaired HGF-induced dextran endocytosis (Fig. 4E), whereas the overexpression of wt-MAP4K4 alone was sufficient to increase endocytic activity to a level similar to GF-stimulated cells (Fig. 4F). Together, these data indicate the function of MAP4K4 in vesicle trafficking and endocytosis to control the up-take of macromolecules into the tumor cells.

MAP4K4 promotes integrin activation on endosomes

Cell migration requires proper binding to extracellular matrix proteins through the family of integrin adhesion receptors and their coordinated turnover through the endocytic machinery (Ridley, Schwartz et al. 2003; Maritzen, Schachtner et al. 2015). The adhesion to extracellular matrix (ECM) proteins causes the internalization of activated ß1 integrins, which accumulate in an activated state on endosomes to promote adhesion and pro-migratory signaling through focal adhesion kinase (FAK) (Alanko, Mai et al. 2015). Therefore, we tested whether MAP4K4 control of global macromolecular uptake through endocytic processes (Fig. 4D-F) is coupled to integrin activation. We quantified the levels of intracellular activated ß1 in cells seeded onto coll I using confocal IFA. Since passage of activated integrins through Rab7-positive late endosomes is one mechanism of integrin recycling (Dozynkiewicz, Jamieson et al. 2012), we monitored abundance of active ß1 integrins at the level of late endosomes. We detected significant decreases in ß1 integrin activation in cells treated with Dyngo 4a, a dynamin inhibitor preventing activity-dependent bulk endocytosis in neurons (McCluskey, Daniel et al. 2013). Interestingly, we found that MAP4K4 is also involved in bulk up-take of activated ß1 integrins, as impairing MAP4K4 functions either by C29 treatment or by siRNA-mediated depletion of MAP4K4 caused a significant drop of activated ß1 integrin in MB cells (Fig. 5A). Since our studies focus particularly on cancer cell migration in 3D environment, where leading edge invasive protrusions play a major role during matrix invasion, we monitored the effect of MAP4K4 depletion on HGF-driven ß1 activation in lamellipodia. HGF stimulation causes a significant increase in active ß1 integrin in lamellipodia of cells migrating inside collagen gels (Fig. 4B). This increase is completely abrogated in MAP4K4-depleted cells (Fig. 4B). To confirm that ß1 integrin is necessary for collagen invasion and migration, we prevented ß1 integrin function using either blocking anti-Iß1 antibody (Fig. 5C) or by siRNA-mediated depletion (Fig. 5D). ß1 integrin blockade using anti-Iß1 caused a significant reduction in 3D cell migration. Frequency distribution analysis revealed that while the majority of cells migrated less efficiently, a considerable number still infiltrated longer distances. (Fig. 5C, right). This is probably due to incomplete antibody blockade of Iß1, as si-Iß1 reverted collagen invasion induced by HGF. One relevant downstream function of Iß1 is the activation of FAK on endosomes, where among many other proteins MAP4K4 was found as well (Alanko, Mai et al. 2015). We therefore tested whether reduced Iß1 activation in cells with impaired MAP4K4 function also causes reduced FAK activation. We tested the levels of activated FAK of MB sgC and sgM4K4 cells embedded in collagen gels by IB using anti phospho-Y397-FAK antibody. We found pFAK levels in cells with impaired MAP4K4 function significantly reduced (Fig. 5 E) and this level of reduced pFAK was not increased in the presence of HGF.
Taken together, these data show that MAP4K4 is required for integrin activation on endosomes in response to collagen adhesion and that this MAP4K4-dependent Iß1 activation is required for migration and invasion and for the activation of FAK.

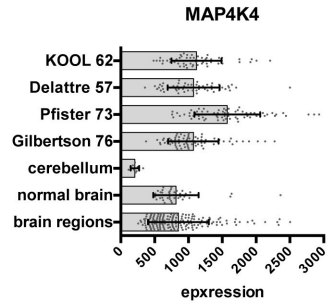
MAP4K4 controls c-Met function

The receptor tyrosine kinase and proto-oncogene c-Met can promote migration and invasion in MB and other solid tumors (Li, Lal et al. 2005; Provencal, Labbe et al. 2009; Trusolino, Bertotti et al. 2010; Sierra and Tsao 2011; Guessous, Yang et al. 2012; Santhana Kumar, Tripolitsioti et al. 2015). c-Met functions are also regulated at the level of receptor endocytosis and c-Met signaling from endocytic vesicles promotes growth, survival and cell migration (Barrow-McGee and Kermorgant 2014). Consistently, pharmacological inactivation of dynamin-dependent endocytosis using Dyngo 4a impaired c-Met activation, caused reduced activation of ERK and repressed FAK activation in MB cells (Fig. 6A). We therefore tested whether MAP4K4 control of endocytic activity (Fig. 4) could also affect HGF-induced c-Met and ERK activation. We found that MAP4K4 depletion caused a marked reduction of HGF-induced activation of c-Met and of downstream ERK

signaling (Fig. 6B), whereas steady state c-Met phosphorylation was not affected or rather increased. Consistent with decreased levels of activated FAK observed in MAP4K4-depleted cells embedded in collagen, we also found FAK activation in response to HGF impaired in the absence of MAP4K4 (Fig. 6B). The inhibition of dynamin-dependent endocytosis causes a significant reduction in intracellular activated c-Met, which in control cells accumulated on vesicular structures in the cytoplasm. (Fig. 6C). We therefore tested whether the reduced global c-Met activation in MAP4K4-depleted cells could be associated with reduced trafficking of activated c-Met or maintenance in vesicles. Cortical F-actin was increased in HGF-stimulated sgControl cells and the appearance of pc-Met-decorated vesicles in the cytosol was apparent. In contrast, sgM4K4 cells displayed reduction of cortical F-actin and a striking decrease in pc-Met-positive vesicles (Fig. 6D). We quantified the number of pc-Met-positive vesicles under all conditions and measured their size using Imaris (Fig. 6C and D, lower). This quantification showed that intracellular vesicle-associated pc-Met was significantly reduced when endocytic activity was impaired or MAP4K4 functions repressed. This experiment furthermore revealed trafficking of activated c-Met through intracellular compartments and that either activation or internalization of c-Met is impaired when MAP4K4 is absent. To determine whether altered internalization of c-Met could explain reduced levels of activated c-Met inside the cells, we quantified HGF-induced c-Met internalization after surface biotinylation. Comparison of control and HGF stimulation revealed that raising the temperature from 0°C to 37 alone was sufficient to cause marked c-Met internalization within 30 min. HGF stimulation further enhanced this effect (Fig. 6E). In the absence of MAP4K4, both temperature shift-induced and HGF-dependent c-Met uptake was completely blocked (Fig. 6E). Taken together these data strongly suggest the implication of MAP4K4 in the control of HGF signaling by modulating up-take and trafficking of its receptor c-Met.

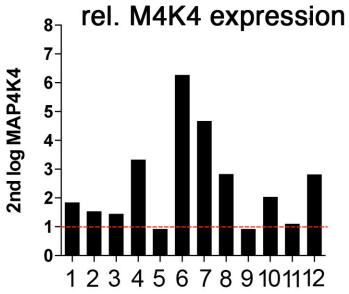
Fig. 1

A



	Kool 62	Delattre 57	Pfister 73	Gilbertson 76
cerebellum	P<0.001	P<0.001	P<0.001	P<0.001
normal brain	P<0.01	P<0.05	P<0.001	P<0.01
brain regions	P<0.01	P<0.05	P<0.001	P<0.01

B



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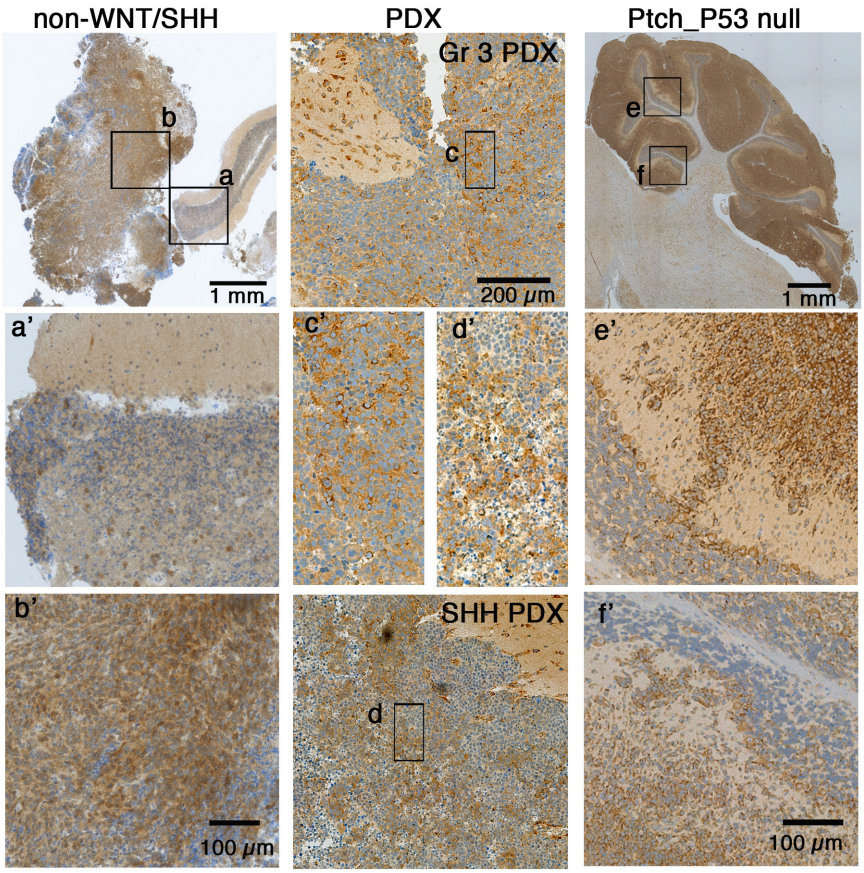

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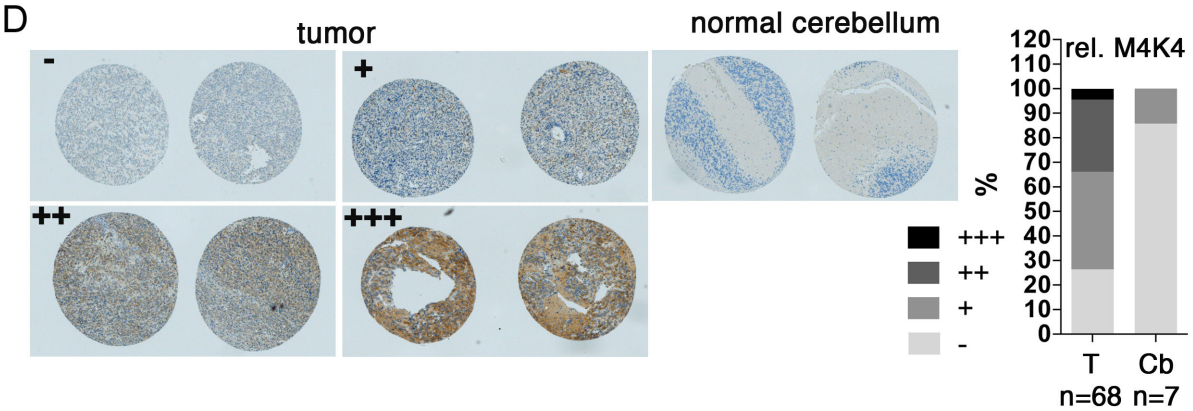


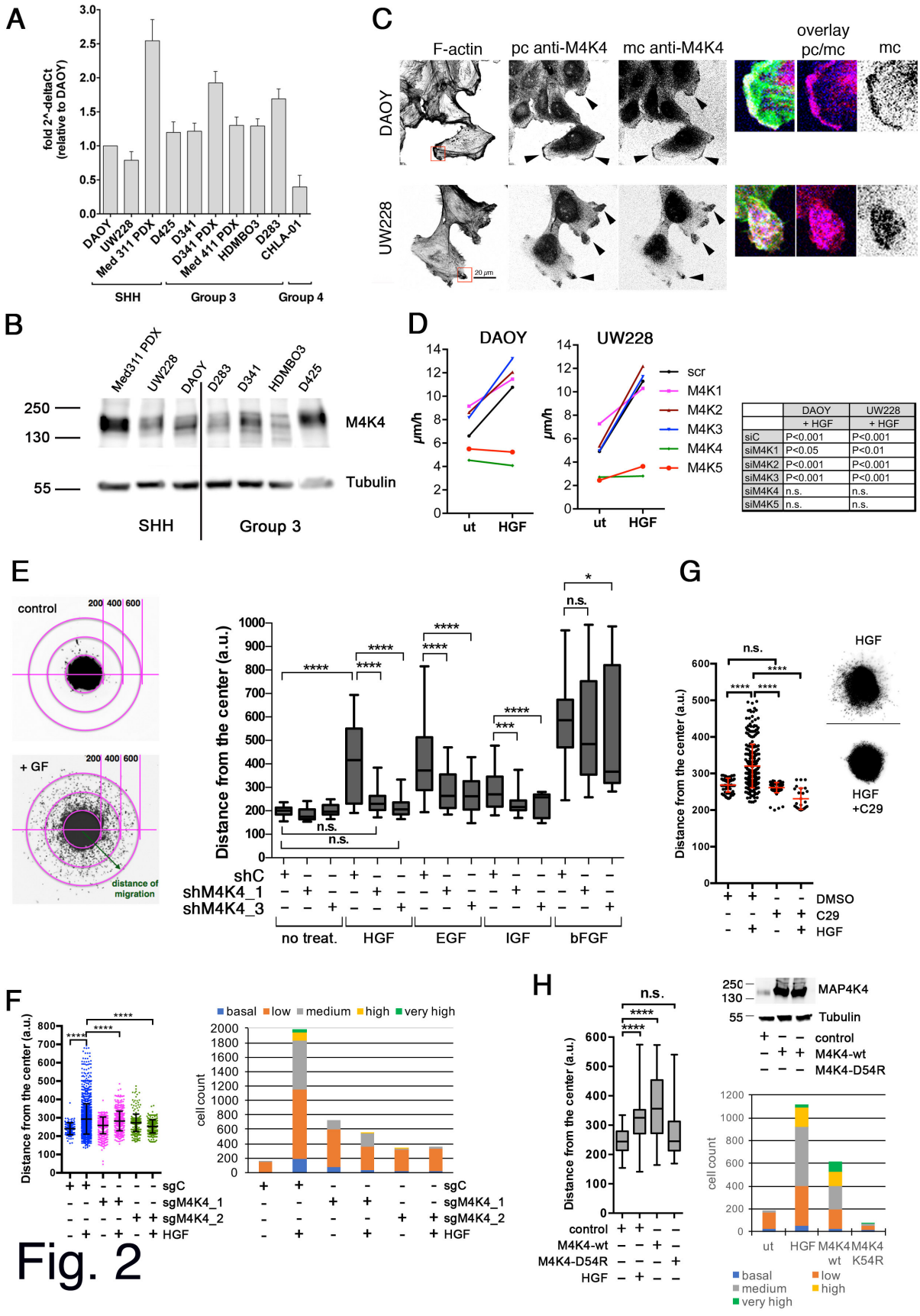
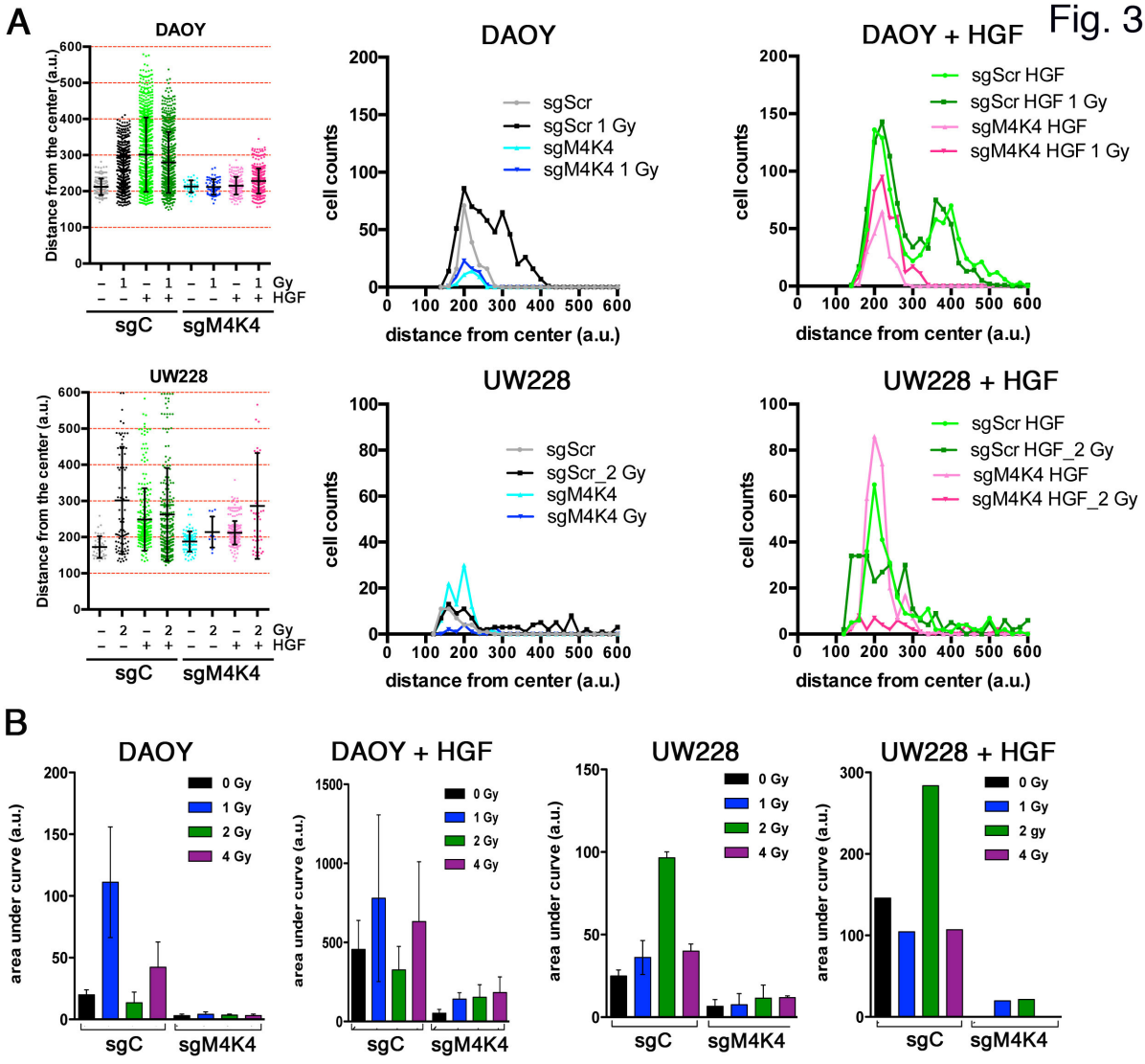

A
fold 2^-deltaCt (relative to DAOY)
DAOY
UW228
Med 311 PDX
D425
D341
D341 PDX
Med 411 PDX
HDMBO3
D283
CHLA-01
SHH
Group 3
Group 4
B
Med311 PDX
UW228
DAOY
D283
D341
HDMBO3
D425
250
130
55
M4K4
Tubulin
SHH
Group 3
C
F-actin
pc anti-M4K4
mc anti-M4K4
overlay pc/mc
mc
DAOY
UW228
20 µm
D
DAOY
UW228
µm/h
ut
HGF
scr
M4K1
M4K2
M4K3
M4K4
M4K5
DAOY + HGF
UW228 + HGF
siC P<0.001 P<0.001
siM4K1 P<0.05 P<0.01
siM4K2 P<0.001 P<0.001
siM4K3 P<0.001 P<0.001
siM4K4 n.s. n.s.
siM4K5 n.s. n.s.
E
control
+ GF
200 400 600
distance of migration
Distance from the center (a.u.)
shC
shM4K4_1
shM4K4_3
no treat.
HGF
EGF
IGF
bFGF
n.s.
G
Distance from the center (a.u.)
HGF
HGF +C29
DMSO
C29
HGF
F
Distance from the center (a.u.)
sgC
sgM4K4_1
sgM4K4_2
HGF
basal
low
medium
high
very high
cell count
H
Distance from the center (a.u.)
control
M4K4-wt
M4K4-D54R
HGF
MAP4K4
Tubulin
ut
HGF
M4K4 wt
M4K4 K54R

Fig. 2

Fig. 3


A
DAOY
Distance from the center (a.u.)
Gy
HGF
sgC
sgM4K4
DAOY
cell counts
sgScr
sgScr 1 Gy
sgM4K4
sgM4K4 1 Gy
distance from center (a.u.)
DAOY + HGF
sgScr HGF
sgScr HGF 1 Gy
sgM4K4 HGF
sgM4K4 HGF 1 Gy
UW228
sgScr_2 Gy
sgM4K4 Gy
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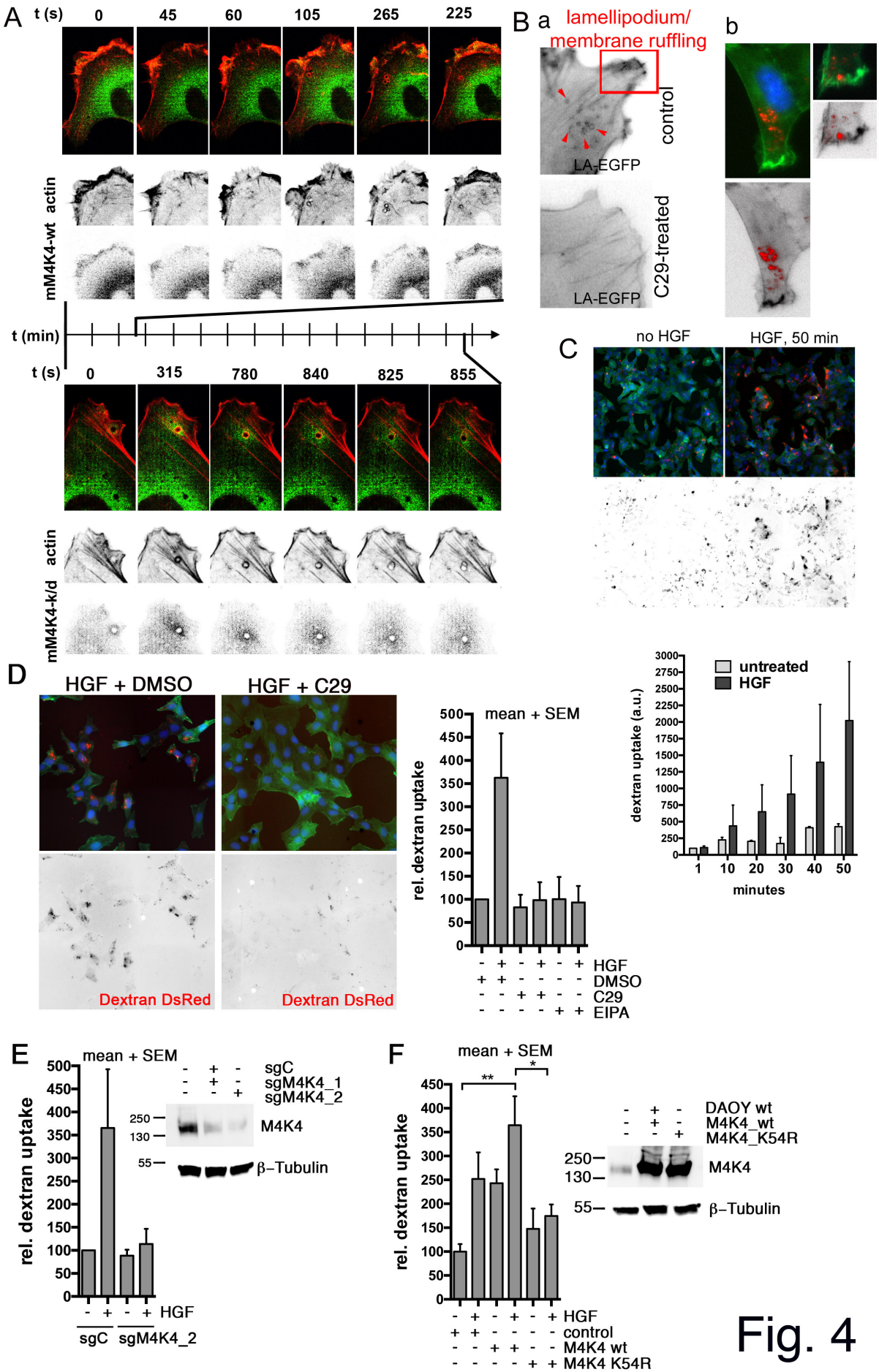
B
area under curve (a.u.)
0 Gy
1 Gy
2 Gy
4 Gy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0
45
60
105
265
225
mM4K4-wt
actin
t (min)
t (s)
0
315
780
840
825
855
mM4K4-k/d
actin
B a
lamellipodium/
membrane ruffling
control
LA-EGFP
C29-treated
LA-EGFP
b
C
no HGF
HGF, 50 min
D
HGF + DMSO
HGF + C29
Dextran DsRed
Dextran DsRed
mean + SEM
rel. dextran uptake
HGF
DMSO
C29
EIPA
untreated
HGF
dextran uptake (a.u.)
minutes
E
mean + SEM
rel. dextran uptake
sgC
sgM4K4_1
sgM4K4_2
M4K4
β–Tubulin
HGF
sgC
sgM4K4_2
F
mean + SEM
rel. dextran uptake
DAOY wt
M4K4_wt
M4K4_K54R
M4K4
β–Tubulin
HGF
control
M4K4 wt
M4K4 K54R

Fig. 4

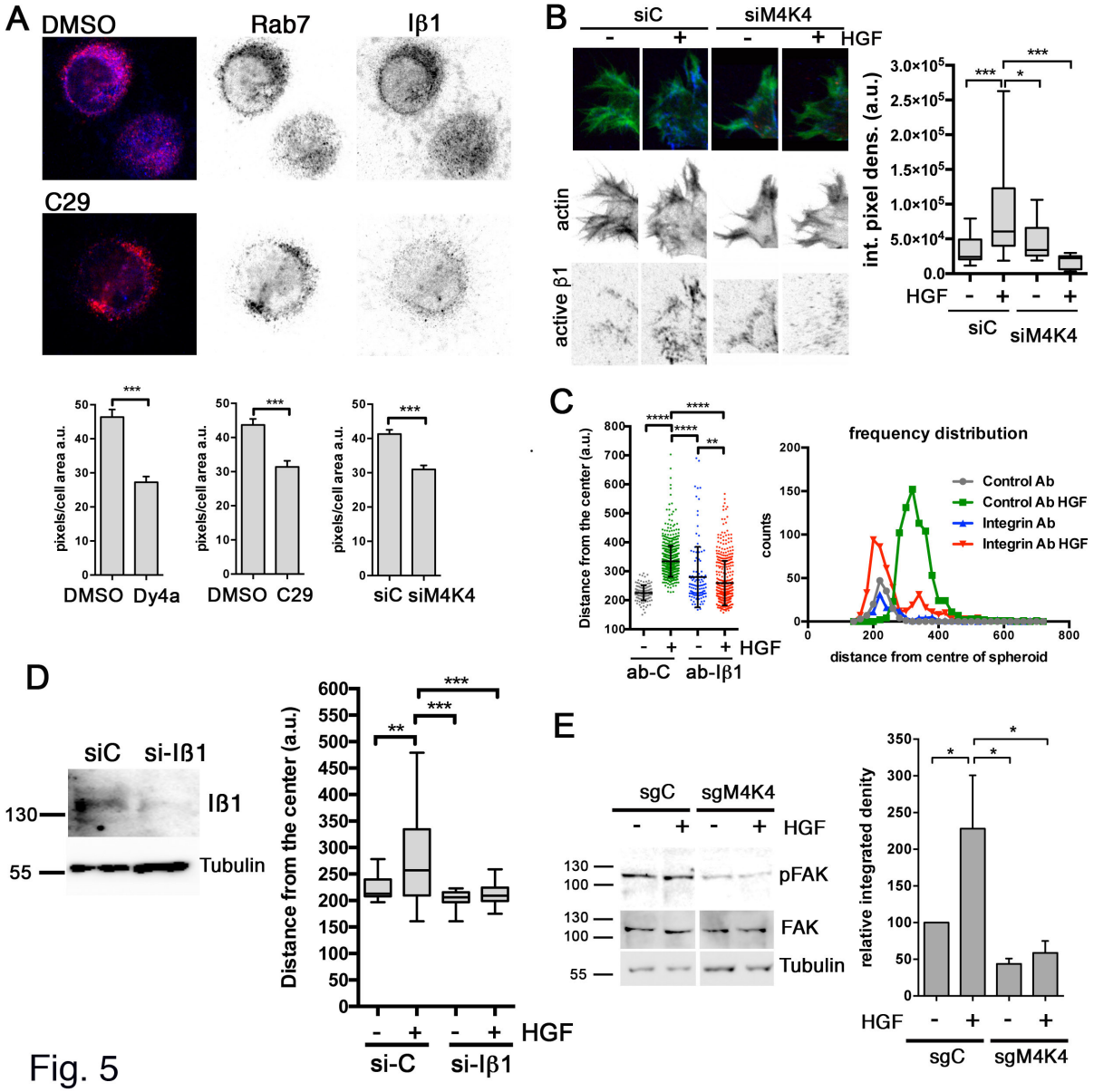

A
DMSO
Rab7
Iβ1
C29
pixels/cell area a.u.
DMSO Dy4a
DMSO C29
siC siM4K4
B
siC
siM4K4
HGF
actin
active β1
int. pixel dens. (a.u.)
C
Distance from the center (a.u.)
ab-C ab-Iβ1
frequency distribution
Control Ab
Control Ab HGF
Integrin Ab
Integrin Ab HGF
counts
distance from centre of spheroid
D
siC si-Iβ1
130
55
Iβ1
Tubulin
Distance from the center (a.u.)
si-C si-Iβ1
E
sgC sgM4K4
pFAK
FAK
Tubulin
relative integrated denity

Fig. 5

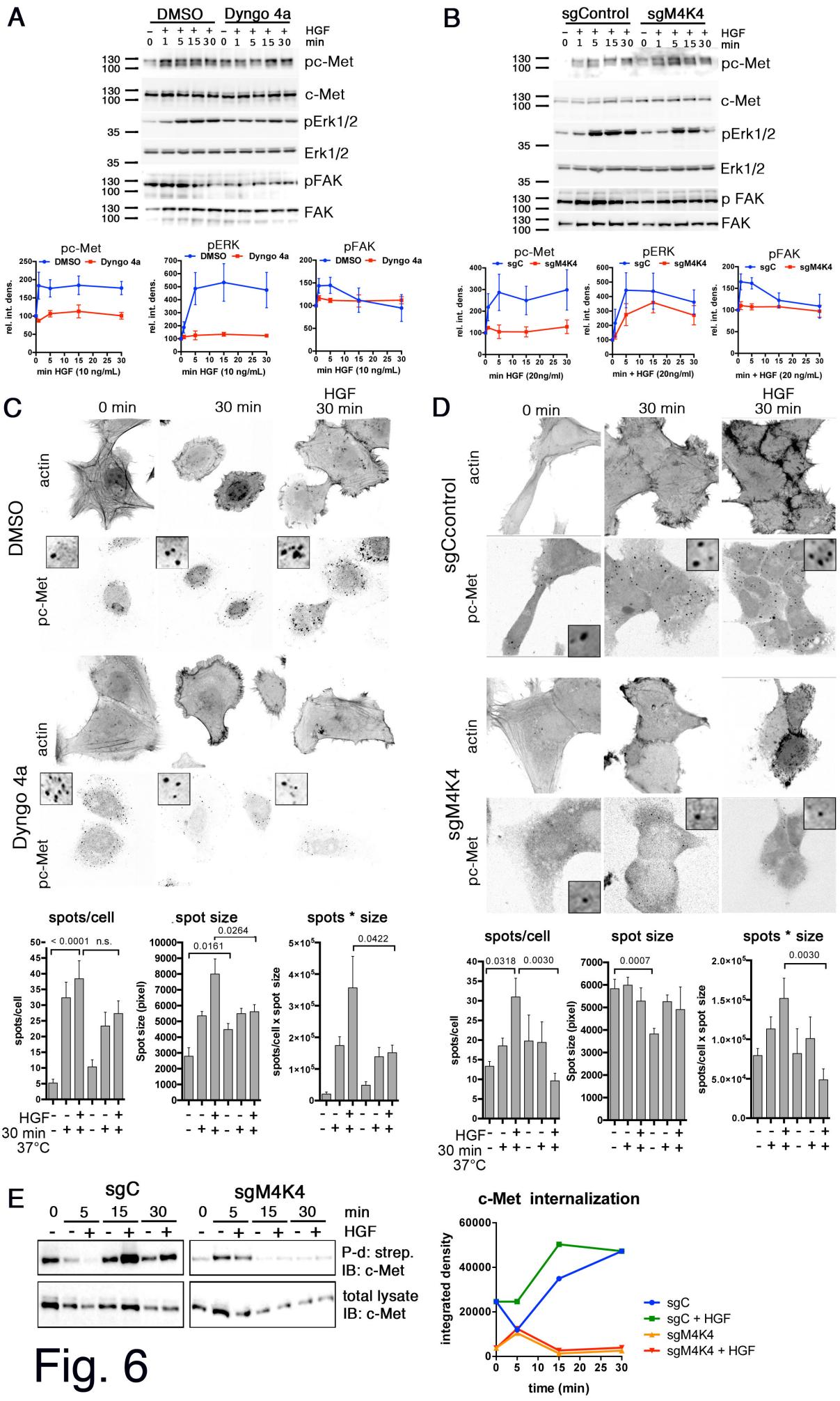

A
DMSO
Dyngo 4a
HGF
min
pc-Met
c-Met
pErk1/2
Erk1/2
pFAK
FAK
pc-Met
pERK
pFAK
rel. int. dens.
min HGF (10 ng/mL)
B
sgControl
sgM4K4
p FAK
sgC
sgM4K4
min HGF (20ng/ml)
min + HGF (20ng/ml)
min + HGF (20 ng/mL)
C
0 min
30 min
HGF 30 min
DMSO
Dyngo 4a
actin
pc-Met
spots/cell
spot size
spots * size
Spot size (pixel)
spots/cell x spot size
< 0.0001
n.s.
0.0161
0.0264
0.0422
HGF
30 min
37°C
D
sgCcontrol
sgM4K4
0.0318
0.0030
0.0007
0.0030
E
sgC
sgM4K4
min
P-d: strep.
IB: c-Met
total lysate
IB: c-Met
c-Met internalization
integrated density
time (min)
sgC
sgC + HGF
sgM4K4
sgM4K4 + HGF

Fig. 6

Fig. S1

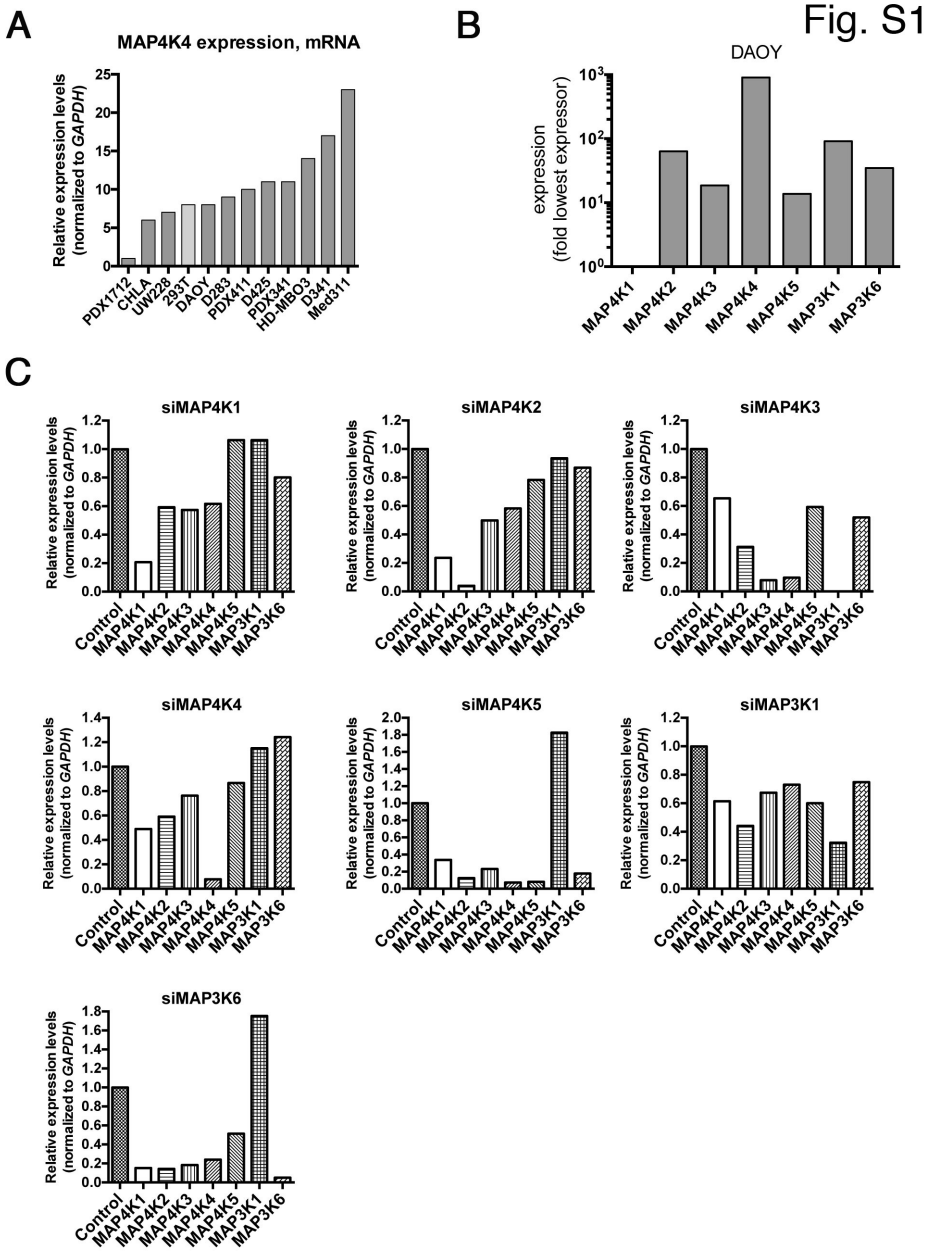

A
MAP4K4 expression, mRNA
Relative expression levels (normalized to GAPDH)
PDX1712
CHLA
UW228
293T
DAOY
D283
PDX411
D425
PDX341
HD-MBO3
D341
Med511
B
DAOY
expression (fold lowest expressor)
MAP4K1
MAP4K2
MAP4K3
MAP4K4
MAP4K5
MAP3K1
MAP3K6
C
siMAP4K1
siMAP4K2
siMAP4K3
siMAP4K4
siMAP4K5
siMAP3K1
siMAP3K6
Relative expression levels (normalized to GAPDH)
Control
MAP4K1
MAP4K2
MAP4K3
MAP4K4
MAP4K5
MAP3K1
MAP3K6

Fig. S2

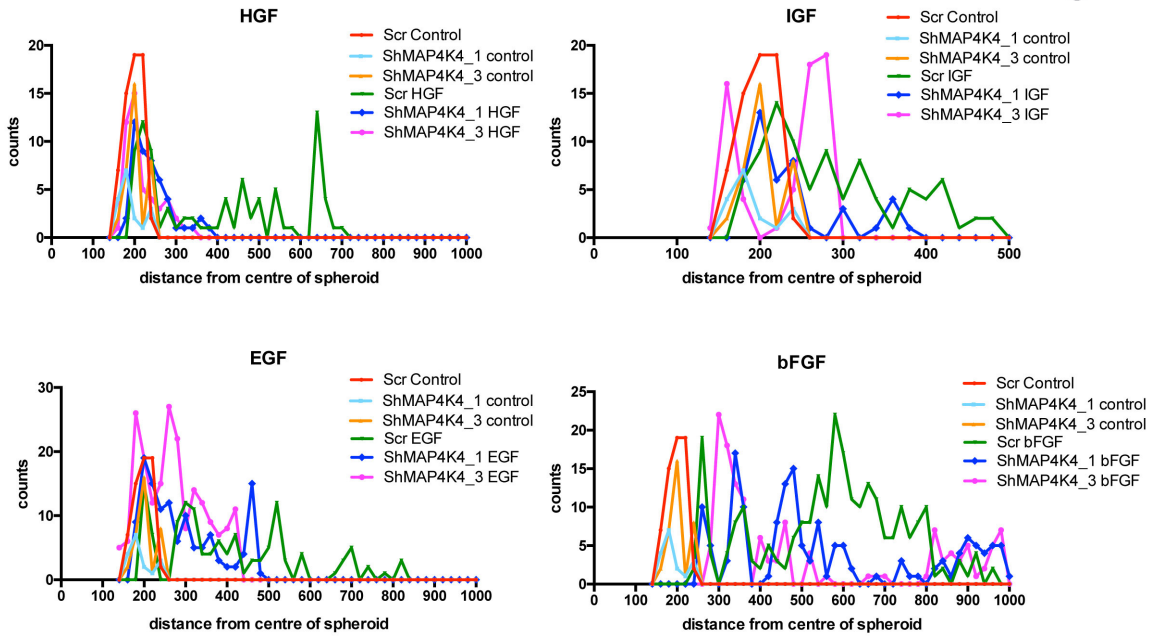

HGF
Scr Control
ShMAP4K4_1 control
ShMAP4K4_3 control
Scr HGF
ShMAP4K4_1 HGF
ShMAP4K4_3 HGF
counts
distance from centre of spheroid
IGF
Scr Control
ShMAP4K4_1 control
ShMAP4K4_3 control
Scr IGF
ShMAP4K4_1 IGF
ShMAP4K4_3 IGF
counts
distance from centre of spheroid
EGF
Scr Control
ShMAP4K4_1 control
ShMAP4K4_3 control
Scr EGF
ShMAP4K4_1 EGF
ShMAP4K4_3 EGF
counts
distance from centre of spheroid
bFGF
Scr Control
ShMAP4K4_1 control
ShMAP4K4_3 control
Scr bFGF
ShMAP4K4_1 bFGF
ShMAP4K4_3 bFGF
counts
distance from centre of spheroid

Fig. S3

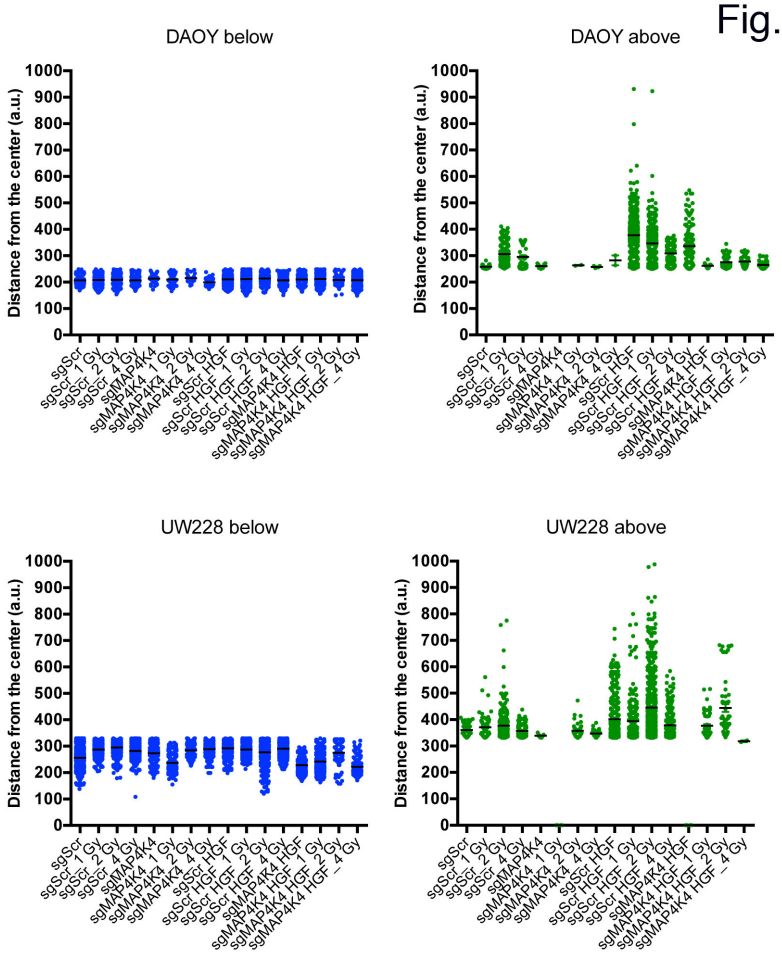
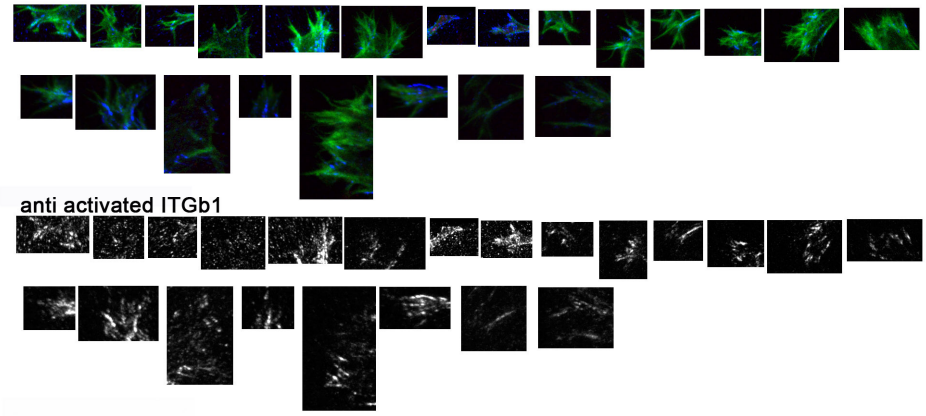
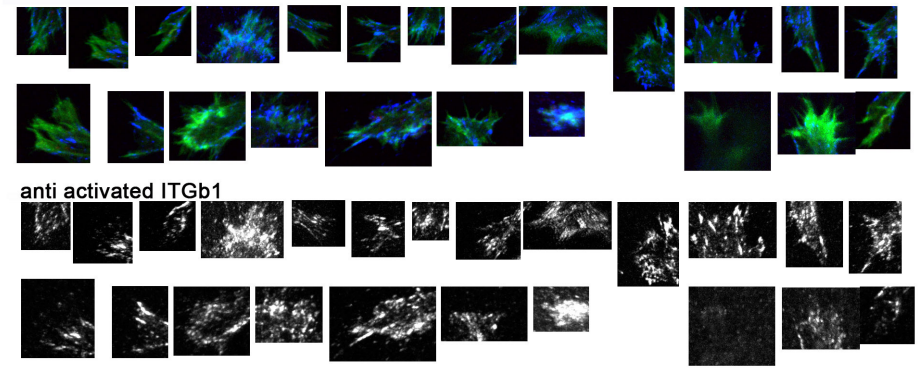

DAOY below
DAOY above
UW228 below
UW228 above
Distance from the center (a.u.)
0
100
200
300
400
500
600
700
800
900
1000
sgScr
sgScr 1 Gy
sgScr 2 Gy
sgScr 4 Gy
sgMAP4K4
sgMAP4K4 1 Gy
sgMAP4K4 2 Gy
sgMAP4K4 4 Gy
sgScr HGF
sgScr HGF 1 Gy
sgScr HGF 2 Gy
sgScr HGF 4 Gy
sgMAP4K4 HGF
sgMAP4K4 HGF 1 Gy
sgMAP4K4 HGF 2 Gy
sgMAP4K4 HGF 4 Gy

Fig. S4

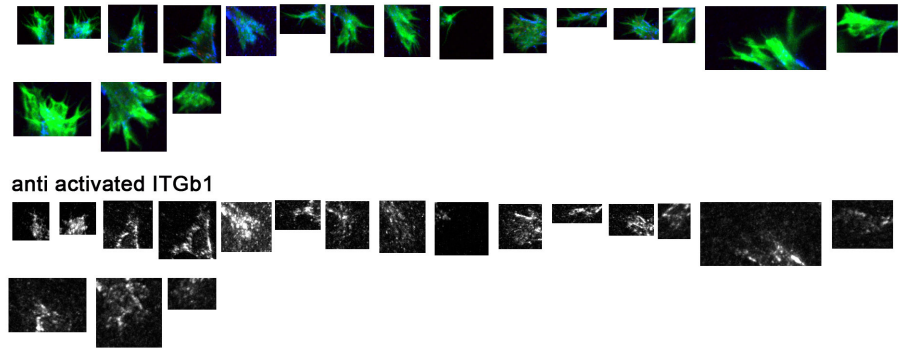
sgScr



sgScr + HGF



sgMAP4K4



sgMAp4K4 + HGF

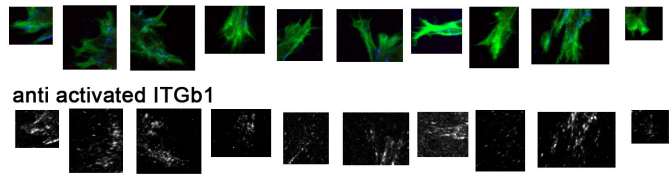


Fig. S5

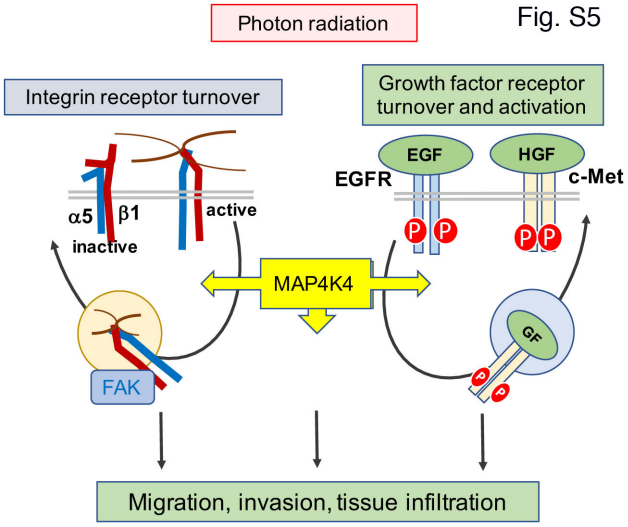

Photon radiation
Integrin receptor turnover
Growth factor receptor turnover and activation
α5
β1
inactive
active
EGF
HGF
EGFR
c-Met
MAP4K4
FAK
GF
Migration, invasion, tissue infiltration

Figure legends

Fig. 1

MAP4K4 is overexpressed in in MB tumors. A) MAP4K4 mRNA U133P Affymetrix gene chip micro array expression levels in normal tissue samples (cerebellum and brain regions) versus MB tumors. Statistical significance of difference is indicated in table below horizontal bar-dot plot. **B)** qRT-PCR quantification of MAP4K4 mRNA expression levels in 12 MB patient samples from the University Children's Hospital Zurich. Red line indicates 2-fold increase of MAP4K4 expression levels compared to normal cerebellum control. **C)** Anti-MAP4K4 IHC analysis in a non-WNT/non-SHH MB primary tumor, a G3 MB PDX and a Ptch/P53 null SHH model of MB a' – f' are 4x magnification of boxed areas. a' is healthy cerebellar tissue, and b' is tumor tissue. c' and d' are different zones in same tumor. e' and f' show MAP4K4-positive neoplastic cells infiltrating the granular layer. **D)** MAP4K4 expression in MB tumor tissue microarray of MB tumor (T) and cerebellum tissue (Cb) samples and H score quantification.

Fig. 2

MAP4K4 promotes migration and invasion. Analysis of MAP4K4 mRNA **(A)** and protein **(B)** expression in different MB cell lines and PDX models by qRT-PCR and immunoblot, respectively. **C)** IFA of MAP4K4 subcellular localization in migrating DAOY and UW228 cells. Signals of polyclonal (pc) and monoclonal (mc) anti-MAP4K4 were compared. Arrowheads indicate MAP4K4 localized in the lamellipodia. Magnificiations are 4x of red boxed areas. **D)** XY plot shows comparison of the speeds of single cells (n=30) transfected with siRNAs against the indicated MAP4 kinases without or with 20 ng/mL HGF. **E)** left: Schematic representation of cell dissemination in collagen I (spheroid invasion assay, SIA) without or with growth factor (GF). Purple concentric circles indicate variable ranges of dissemination from the spheroid center. Right: SIA of DAOY cells expressing control or one of two different *MAP4K4*-targeting shRNAs in the absence or presence of different GFs (HGF: 20 ng/mL, EGF: 30 ng/mL, IGF: 20 ng/mL, bFGF: 100 ng/mL). **F)** SIA of cells with CRISPR/CAS9-mediated knock-down of MAP4K4 without or with HGF. Bar graphs show frequency distribution for each condition (dissemination ranges: basal: 100 – 200, low: 200 – 300, intermediate: 300 – 400, high: 400 – 500, very high: 500+). **G)** SIA of DAOY cells treated without or with C29 and HGF. Images to the right are representative images of quantification. **H)** SIA with DAOY control, or DAOY ectopically expressing either MAP4K4 wild-type (wt), or MAP4K4 kinase-dead mutant (K54R). IB shows relative MAP4K4 expression in control and overexpressing cells. Bar graphs shows frequency distribution for each condition (dissemination ranges: basal: 100 – 200, low: 200 – 300, intermediate: 300 – 400, high: 400 – 500, very high: 500+). . $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (1way Anova).

Fig. 3

A) Left: Representative dot plots of SIA of DAOY and UW228 cells (shControl and sgMAP4K4_2) exposed to one (DAOY) or two (UW228) Gray (Gy) irradiation after embedding in collagen. SIA was performed immediately after irradiation either in the absence or presence of 20 ng/mL HGF as indicated. Right: frequency distributions of the dot plots shown. **B)** Quantification of the area under the curve (AUC) of the frequency distributions with a cut-off of higher than 250 from the spheroid center. Mean and SEM of three (DAOY) or two (UW228) or one (UW228 + HGF) independent experiments are shown.

Fig. 4

MAP4K4 promotes endocytic uptake. A) Still images of time lapse movies of DAOY cells transfected with vectors expressing mM4K4 wild-type or mM4K4D152N (k/d) kinase dead mutant. LA-mCherry in red, EGFP-mM4K4 in green. Inverted grey scale images show actin and MAP4K4. **B)** Left: same as in A) in DAOY cells treated with DMSO or MAP4K4 inhibitor C29, LA-EGFP in grey scale. Arrows point to trafficking vesicles. Right: IFA analysis of cells exposed to 70 kDa DS red dextran for 30 min. **C)** 70 kDa DS red dextran uptake in DAOY cells -/+ HGF 20 ng/mL. Upper: IFA showing representative images of DAOY cells 50 min after dextran exposure. Lower: quantification of HGF-dependent dextran uptake. **D)** 70 kDa DS red dextran uptake in DAOY cells treated with either DMSO, EIPA or C29. Left: representative images of dextran uptake -/+ C29. Green: F-actin, blue: DNA, Red: Dextran. Right: quantification of uptake 30 min after dextran addition. **E**

& F) Quantification of TMR Dextran internalization after transient downregulation of MAP4K4 using siRNA (E) or overexpression of hM4K4 wt or hM4K4K54R kinase-dead MAP4K4 (F) -/+ HGF. Quantifications presented as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 (1way Anova).

Fig. 5.
MAP4K4 promotes integrin signaling. A) Quantification of activated ITGB1 in DAOY cells plated on Coll I -/+ Dyngo 4a (Dy4a) or C29 or after MAP4K4 downregulation by siRNA. Representative confocal cross-sections at level of Rab7 are shown. Green: F-actin, blue: activated ITGB1, red: Rab7. Bar graphs depict quantification of activated ITGB1 on Rab7-positive endosomes upon dynamin (Dyngo (McCluskey, Daniel et al. 2013)) or MAP4K4 (C29) inhibition or after transient depletion of MAP4K with siRNA. Mean and SEM of pooled data from 2 (compounds C29 and Dyngo 4a) and 3 (siM4K4) independent experiments are shown **B)** Left: representative images of activated ITGB1 in lamellipodia of DAOY cells invading Coll I matrix in siScr (siC) or siMAP4K4 cells. Right: quantification of activated ITGB1 in lamellipodia. At least 10 lamellipodia were analyzed per condition (See Fig. S4). **C)** Dot plot (left) and frequency distribution (right) of blocking anti-ITGB1 or control ab effect on Coll I invasion in SIA and in response to HGF (20 ng/mL). **D)** Immunoblot and quantification of FAK phosphorylation in Coll I-embedded DAOY sgC and sgM4K4 cells.

Fig. 6
MAP4K4 promotes internalization and activation of cMet. A & B) Top: IB analysis of c-Met, Erk1/2 and FAK phosphorylation in response to HGF stimulation in the absence or presence of 10 μM Dyngo 4a. (A) or MAP4K4 depletion (B). Bottom: XY line plots of integrated pixel densities of phospho bands from 3 experiments against time in min after HGF stimulation. **C)** Upper: confocal IFA of PLL adhering DMSO or Dyngo 4a-treated cells. pc-Met before (0 min) and after raising temperature from 0° to 37°C in the absence or presence of HGF. Insets show 4x magnification of spots. Bottom: bar diagram of single cell analyses of spots/cell, spot size and spots * size (n=13, mean and SD). **D)** As C) but either sgC or sgM4K4_2 cells were used. **E)** Left: biotinylation assay in sgC or sgM4K4_2 cells -/+ HGF. Right: quantification of internalized biotinylated c-Met relative to total c-Met. *p < 0.05, **p < 0.01, ***p < 0.001 (1way Anova).

Supplementary Information:
Figure S1:
A) qRT-PCR quantification of mRNA expression levels of MAP4K4 in different MB lines and in HEK293T cells. **B)** qRT-PCR quantification of the MAP4K1-5, MAP3K1 and MAP3K6. Normalization using GAPDH expression levels in DAOY cells. **C)** qRT-PCR quantification of siRNA effects on target MAP4Ks and MAP4K1-5, MAP3K1 and MAP3K6. individual siRNA transient downregulation in DAOY cells.

Figure S2:
Frequency distribution curves (distance plotted against cell counts) derived from the spheroid invasion assay dot plots shown in 2E, right panel. MAP4K4 was depleted with two different shRNAs and SIA was performed in the absence or presence of growth factor (HGF: 20 ng/mL, EGF: 30 ng/mL, IGF: 20 ng/mL, bFGF: 100 ng/mL) in DAOY MB cells.

Figure S3:
Irradiation promotes cell invasion in a MAP4K4-dependent manner in Medulloblastoma. Quantification of the Area under curve of frequency distributions of SIA with DAOY **(Top)** and UW228 **(Bottom)** cells after different irradiation dosages (0, 1, 2, 4 Gy). MAP4K4 was depleted by shRNA and SIA was performed in the absence of presence of HGF (20 ng/ml). The cut-off was defined as above 90% of the control non-irradiated cell population. Data are the mean and SEM of three independent experiments.

Figure S4
MAP4K4 promotes HGF-induced integrin activation in lamellipodia. Confocal microscopy images showing activated ß1 integrin (anti-activated ITGB1, 12G10 antibody) in lamellipodia of collagen-embedded

DAOY sgScr and sgMAP4K4 cells without or with HGF (20 ng/ml) stimulation for 24h. Blue: Activated ß1 integrin. Green: Actin.

Figure S5

Scheme depicting MAP4K4 implication of integrin α51ß1 and c-Met receptor turnover and activation for establishment of the pro-metastatic phenotype.

Discussion:

Our data indicate that the up-regulation of MAP4K4 in MB controls relevant aspects of the metastatic behavior of the tumor cells by promoting productive turnover of integrin ß1 and the RTK c-Met in response to growth factor stimulation. GF-induced receptor downstream signaling and invasive motility of the tumor cells depend on the expression and activity of MAP4K4. We propose that the mechanisms underlying these MAP4K4 functions are linked at the level of endocytic turnover and vesicle trafficking. This novel activity of the tumor-associated MAP4K4 identified a potentially druggable mechanism to restrict tumor cell migration and invasion by interfering with the endocytic processing of adhesion and growth factor singaling.

We detected both increased MAP4K4 mRNA as well as protein levels in tumors compared to the normal cerebellum controls. Interestingly, increased MAP4K4 staining was also detected in tumor cells of the Ptch-p53null mouse model invading the healthy tissues (Fig. 1). Thus, similar to other solid tumors such as glioblastoma, pancreatic ductal adenocarcinoma, lung cancer and gastric cancer, MAP4K4 is overexpressed in the cerebellar tumor tissue. High MAP4K4 mRNA expression was also detected in glioma, which confirms the intriguingly increased brain tumor-specific MAP4K4 expression levels noted a while ago (Wright, MCB 2003). Although MAP4K4 expression in tissue-infiltrating cells of Ptch-p53null MB indicated the potential contribution of MAP4K4 to tumor cell invasion, functional analysis will be necessary to clarify the potential impact of MAP4K4 on tissue infiltration in this model. The increased MAP4K4 expression in at least 30% of the 68 human MB tissue samples analyzed indicates the potential diagnostic relevance of MAP4K4 for the clinic. However, to use anti-MAP4K4 IHC analysis as a diagnostic tool, the biological significance of MAP4K4 function in MB pathogenesis and its association with clinical parameters needs to be further clarified.

5. Discussion

In the present study, we have examined the function of the Ser/Thr MAP4K4 in the context of Medulloblastoma (MB) dissemination. We have shown that MAP4K4 expression is upregulated in Medulloblastoma tumors compared to normal healthy tissues. Furthermore, we have developed appropriate biological tools to analyze MAP4K4 function in Medulloblastoma (MB). These tools enabled us to reveal that MAP4K4 comprises an essential mediator of actin dynamics as well as endocytic uptake in MB cells and that MAP4K4-dependent processes promote pro-invasive functions in MB.

5.1 MAP4K4 is overexpressed in Medulloblastoma

Previous studies had demonstrated MAP4K4 involvement in tumorigenesis, mainly through observations that MAP4K4 levels were enhanced in different types of cancers and more importantly, in later, more aggressive tumor stages (Badea, Herlea et al. 2008; Liang, Wang et al. 2008; Hao, Chen et al. 2010; Liu, Cai et al. 2011; Qiu, Qian et al. 2012). Nevertheless, with MB being a highly metastatic tumor, MAP4K4 function towards metastatic MB dissemination remained unclear. Thus, one of the first steps we undertook to explore whether MAP4K4 could be implicated in the context of MB invasive functions, was to examine MAP4K4 expression levels in MB tumors compared to samples from the normal cerebellum and normal brain regions. We detected both increased MAP4K4 mRNA as well as protein levels in tumors compared to the normal cerebellum controls (Fig. 1&B, Manuscript 2). Interestingly, increased MAP4K4 staining was also detected in tumor cells of the Ptch-p53null mouse model invading the healthy tissues (Fig. 1C, Manuscript 2). Thus similar to other solid tumors such as glioblastoma, pancreatic ductal adenocarcinoma, lung cancer and gastric cancer, MAP4K4 is overexpressed in the cerebellar tumor tissue. Interestingly, high MAP4K4 mRNA expressoin was also detected in glioma, which confirms the high brain tumor-specifc MAP4K4 expression levels noted a while ago (Wright, Wang et al. 2003). Although the MAP4K4 expression in tissue-infiltrating cells of Ptch-p53null MB indicated the potential contribution of MAP4K4 to tumor cell invasion, functional analysis will be necessary to clarify the potential impact of MAP4K4 on tissue infiltration. The increased MAP4K4 expression in at least 30% of the 68 human MB tissue samples analyzed indicates the potential diagnostic relevance of MAP4K4 for the clinic. However, to use anti-MAP4K4 IHC analysis indeed as a diagnostic tool, the biological significance of MAP4K4 function in MB pathogenesis and its association with clinical parameters needs to be clarified.

5.2 Generation of tools to study MAP4K4 function

MAP4K4 functions were linked to cytoskeletal rearrangements, cell shape and migration control, cell transformation and metastasis in neurons (Teuliere, Gally et al. 2011), adenocarcinoma cells (Baumgartner, Sillman et al. 2006), glioma (Loftus, Yang et al. 2013), epithelial (Lewellyn, Cetera et al. 2013) and endothelial cells (Haas, Bala et al. 2013), keratinocytes (Haas, Bala et al. 2013) and macrophages (Ma and Baumgartner 2014). To define MAP4K4 functions in Medulloblastoma cells, we initially reduced MAP4K4 expression using different stable and transient RNA interference methods.

Interference with siRNA led to our initial observations that MAP4K4 promotes MB cell motility. However, since siRNA effects are temporary and may confer ‘’off target’ effects, I proceeded towards the generation of stable cell lines with doxycycline-inducible expression of short hairpin RNA targeting MAP4K4. The use of these cell lines obviously offered significant advantages in terms of the duration of the effects, however downregulation using shRNA still resulted in only a partial downregulatoin of MAP4K4 (knock-down) and certain levels of kinase activity were still maintained.

Therefore, to completely abrogate MAP4K4 functions I next established the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9) system for MAP4K4 in MB. The CRISPR/Cas9 system was introduced into mammalian organisms four years ago (Cong, Ran et al. 2013) and has become an effective tool for various genome editing purposes. CRISPR/Cas9 system can induce both gain- and loss-of-function mutations, and it causes less off-target effects than conventional RNAi methods. Although we observed a more complete reduction of MAP4K4 protein expression in

CRISPR/CAS9-targeted MAP4K4 MB cells, residual MAP4K4 was still detected, which may be the consequence of mono-allelic knock-down of MAP4K4 only.

As an alternative approach to the genetic depletion of MAP4K4, I also used stable ectopic overexpression of a kinase-inactive MAP4K4 (MAP4K4-K/D) mutant. To explore the effect of increasing MAP4K4 expression, I also established stable MB cell lines overexpressing MAP4K4. The stable lines expressed MAP4K4 at a level approximately 5-fold higher than the endogenous kinase. Although MAP4K4-K/D expression phenocopied some of the effects such as decreased endocytic up-take, it was unable to act as a dominant-negative on others (HGF-induced collagen invasion). This discrepancy could be due to a kinase-independent scaffolding function of MAP4K4 for migration control or due to ineffective competition with the endogenous MAP4K4 molecules. Interestingly, ectopic expression of MAP4K4-wt resulted in increased basal dissemination levels, similar to those observed after HGF stimulation. This indicates that increasing MAP4K4 abundance above endogenous levels causes growth factor-independent activation of pro-migratory signaling and identifies MAP4K4 as a putative oncogene in Medulloblastoma.

We also compared effects of MAP4K4 depletion to the pharmacological inhibition of MAP4K4 using C29, an experimental ATP-competitive MAP4K4 inhibitor developed by Genentech. In most assays, pharmacological inactivation of MAP4K4 using C29 produced more pronounced effects than the genetic depletion approaches. This may be in part due to the only partial efficacy of the targeting approaches. Nevertheless, off-target effects of C29 towards other kinases such as for example Mink1 cannot be excluded (Crawford, Ndubaku et al. 2014).

5.3 MAP4K4 promotes MB cell motility

Others and our group have revealed a striking association of c-Met receptor expression with the SHH MB subgroup (Onvani, Terakawa et al. 2012; Ramaswamy, Remke et al. 2013). Our group also detected increased c-Met expression in DAOY and UW228 cells, which are considered SHH-like MB cell lines (Onvani, Terakawa et al. 2012; Gotschel, Berg et al. 2013) and which are the main cell model of the present study.

Cell motility and invasion experiments in our group had previously established the functional relevance of HGF-c-Met signaling as promoter of MB single cell migration on flat surfaces and inside three-dimensional collagen matrix. These observations complied with previous findings that have reported HGF induction of 3D cell motility in ductal adenocarcinoma, human mammary fibroblasts as well as endometrial carcinoma (Park, Ryu et al. 2003; Doyle, Carvajal et al. 2015; Spina, De Pasquale et al. 2015).

In an number of cell migration experiments, I found that depletion of MAP4K4 using either siRNA or shRNA prevented HGF-induced pro-invasive activities. One of these experiments was the cell scattering assay. HGF, or scatter factor, is characterized by its ability to promote cell scattering (Naldini, Weidner et al. 1991). Cell scattering is a process defined by the detachment of single cells from epithelial cell colonies and their dispersion (scattering). The detached cells exhibit a motile phenotype, continue to migrate, leading thus to a "scatter" phenomenon. The scattering of epithelial cells confers characteristics of the EMT phenotype, such as the loss of epithelial cell-cell junctions and the acquisition of a motile mesenchymal phenotype. Hence, cell-scattering assays have been used for studying EMT phenotype, as well as for detecting factors able to induce migratory cell behavior. In the present study, we used the principle of the cell scattering assay, to confirm MAP4K4 involvement in HGF-induced MB cell motility regulation. In line with our previous findings, we noticed that induced MAP4K4 depletion markedly reduced cell scattering (Fig. 5 & E, manuscript 1). Although the mechanism targeted by MAP4K4 for HGF-induced cell scattering is not known yet, this finding confirms that MAP4K4 mediates HGF-c-Met signaling and leads to increased MB cell migration in 2D in response to HGF, which consists one of the novel findings of the present study. Other RTKs can also activate MAP4K4 and trigger cell migration (Yao, Zhou et al. 1999; Yan, Nehrke et al. 2001; Baumgartner, Sillman et al. 2006). Consistent with the possibility that MAP4K4 acts downstream of activated RTKs, we found that MAP4K4 is mediating pro-migratory signaling downstream of EGF, IGF and bFGF (Fig. 2E, manuscript 2), which we all found to trigger migration and collagen invasion in MB (Kumar, Pillong et al. 2015). However, since MAP4K4-depletion using shRNA only partially blocked bFGF-induced migration, it is

highly likely that in MB cells MAP4K4-dependent and –independent pathways donwstream of RTKs are engaged for migration control and that the relative contribution may depend on the RTK and its immediate downstream effectors.

Cell migration is a complex and dynamic process and is required for a number of basic cellular functions, such as morphogenesis, tissue regeneration, and cancer progression. Our study on MB cell migration in collagen gels demonstrated that MB cells migrate in the mesenchymal mode (Fig. 6, manuscript 1; Fig. S2 (Kumar, Pillong et al. 2015)). Unlike other cancer cells, we could so far not detected rounded/amoeboid motility in MB. Although MAP4K4 ablation does not prevent mesenchymal cell morphology, it caused a marked reduction in F-actin in lamellipodia (Fig. 6, manuscript 2). Since these protrusions are necessary for effective movement of cells inside 3D matrices (Jacquemet, Hamidi et al. 2015), we concluded that one function of MAP4K4 towards migration and invasion control is to facilitate their establishment. MAP4K4 can promote F-actin dynamics and lamellipodia formation by activating the Arp2/3 complex (LeClaire, Rana et al. 2015) and proteins of the Ezrin, Radixin, Moesin family (Baumgartner, Sillman et al. 2006; Vitorino, Yeung et al. 2015). To initiate tumor cell motility, MB cells interact with typical components of the leptomeningeal extracellular matrix (ECM), such as fibronectin, collagen I and collagen IV (Wikstrand, Friedman et al. 1991; Liang, Diehn et al. 2008). Thus, it is also conceivable that the accelerated turnover of β1 integrin-mediated focal adhesions triggered by MAP4K4 recently reported (Yue and Wu 2014; Vitorino, Yeung et al. 2015) is necessary for MB cell invasion. Consistently, using an antibody raised against activated β1 integrin, we observed increased levels of activated β1 integrin in lamellipodia of HGF-stimulated MB cells invading collagen matrix compared to unstimulated control cells. In sgMAP4K4 cells, this increase was ablated, suggesting that MAP4K4 controls β1 integrin activation in a spatially controlled manner. However, it will need to be determined whether decreased integrin activation in the absence of MAP4K4 is the consequence of altered turnover as reported by Yue et al. and Vitorino et al. (Yue and Wu 2014; Vitorino, Yeung et al. 2015).

Taken together, our migration and invasion analyis indicates that the phenomenon of early, rapid and extensive CNS dissemination of MB (Wikstrand, Friedman et al. 1991; Fiorilli, Partridge et al. 2008; Nalla, Asuthkar et al. 2010) could be mediated – at least in part - by MAP4K4 stimulation of promigratory cell functions. Our study furthermore indicates that these could involve - besides the activation of F-actin dynamics reported previously – the accelerated turnover and concomittant activation of RTKs and integrin receptors. Serous chemoattractants, which can abundantly be found within CSF or blood plasma (Rubin, Kung et al. 2003; Dudu, Able et al. 2012) are likely to positively influence the migratory capabilities of the MB cells. In this regard, MAP4K4 transmission of GF signals could further enhance migration and invasion in MB.

5.4 MAP4K4 promotes irradiation-induced cell migration

Apart from the intrinsic promoters of cell migration, treatment-related factors such as photon ionizing radiation (IR), have also been reported to increase MB tumor cell motility. IR has been found to enhance cell migration in sarcoma (Ogata, Teshima et al. 2005), colorectal cancer (Goetze, Scholz et al. 2007), and glioma (Wild-Bode, Weller et al. 2001). In medulloblastoma, preclinical studies have demonstrated increased tumor cell invasiveness through upregulation of urokinase plasminogen activator receptor (uPAR) signaling (Nalla, Asuthkar et al. 2010; Asuthkar, Gondi et al. 2012). In D283 and UW228 medulloblastoma cells, single photon doses of 6 Gy promoted uPAR-dependent Wnt/β-catenin-signaling and consequently increased the neurosphere-forming ability (Asuthkar, Gondi et al. 2012). Moreover, single photon doses of 7 Gy induced expression of α3 , α5 and β1 integrins, leading to enhanced wound healing migration in D283 and DAOY cells (Nalla, Asuthkar et al. 2010). To date, little data has focused on dose-dependent alterations in medulloblastoma cell motility, and none has addressed the potential impact of carbon ion IR.

Since IR contribution to MB cell dissemination remained controversial, we determined IR effects on migration and invasion in DAOY and UW228 MB cell lines. Somewhat counterintuitively, we found that IR

indeed mediated cell migration in both cell lines. More importantly, this effect was prominent in low IR dosages such as 1 - 2 Gy, which corresponds to a single therapeutic radiation dose. Since we had identified MAP4K4 as a critical molecule in MB cell motility regulation, we proceeded to explore its implication in IR-induced cell migration in MB. Therefore we irradiated control as well as MAP4K4-depleted cells and compared the resultant dissemination levels. Interestingly, MAP4K4 decreased IR-induced cell dissemination levels in both cell lines, not only in untreated but also HGF-stimulated cells, suggesting that MAP4K4 is a key regulator of IR-induced cell motility. Taken together, the above findings underscore the relevance of MAP4K4 in the context of MB cell dissemination.

The main mechanism through which conventional radiation therapy targets cancer cells is DNA damage, including DNA dimers, oxidative base damage, intra- and inter-strand crosslinks, and single and double-strand breaks (DSBs). In order to repair the DNA damage, a cell has developed mechanisms for DNA damage sensing, that activate signal transduction pathways, which in turn orchestrate multiple cellular functions for successful damage repair (Wilson 2004). The finding that irradiation causes an increased migratory behavior suggests that besides DNA damage, other activating alterations may occur. More specifically, considering that MAP4K4 promoted IR-induced MB cell migration upon irradiation, one possible explanation could be that irradiation results in DNA alterations that increase the expression and/or secretion of HGF. This could cause increased c-Met signaling whereupon MAP4K4 could mediate cell migration. However, we could not detect increased HGF secretion after irradiation nor any alterations in the expression of c-Met or integrins (data not shown). Therefore, further experiments to test HGF expression and function in irradiated versus non-irradiated cells are required to clarify the mechanisms underlying MAP4K4 control of IR-iduced migration in MB.

Cumulatively, the above observations corroborate previous results that had reported increased MB dissemination after irradiation (Nalla, Asuthkar et al. 2010) and seed the soil for questions regarding the potential objectionable effects on the clinical applications of craniospinal irradiation in Medulloblastoma. Our identification of MAP4K4 as a putative regulator of IR-induced cell migration further highlight its significance as a therapeutic target.

5.5 Unravelling the mechanisms of MAP4K4-mediated functions

Numerous studies have described MAP4K4 role in development (Su, Treisman et al. 1998; Xue, Wang et al. 2001), insulin sensitivity (Su, Treisman et al. 1998; Xue, Wang et al. 2001), systemic inflammation (Aouadi, Tesz et al. 2009), pathogen-dependent oncogenic progression (Haas, Bala et al. 2013; Ma and Baumgartner 2014), vascular inflammation (Flach, Skoura et al. 2015) and cancer (Badea, Herlea et al. 2008; Liang, Wang et al. 2008; Hao, Chen et al. 2010; Liu, Cai et al. 2011; Qiu, Qian et al. 2012). However, the mechanisms as well as the signaling cascades that involve MAP4K4 during Medulloblastoma progression still remain unclear.

Towards the elucidation of the molecular mechanisms through which MAP4K4 exerts its functions in Medulloblastoma, we were initially based on our observations regarding the phenotype of MB cells overexpressiong MAP4K4 wt or K/D mutant. In particular, we noticed that the cells overexpressing MAP4K4 wt presented increased cortical F-actin turnover compared to the cells overexpressing the MAP4K4 K/D. Moreover, the rate of vesicle trafficking as well as the number of the recycled vesicles in the MAP4K4 wt cells appeared to be markedly increased (Fig. 27 & Fig. 5C manuscript 1). Therefore, we concluded that MAP4K4 overexpression coincides with increased cortical F-actin turnover and increased vesicle trafficking. Hence we hypothesized that MAP4K4 could be involved in the regulation of F-actin dependent endocytic processes such as macropinocytosis (Commisso, Davidson et al. 2013).

5.5.1 Regulation of endocytosis

To test this hypothesis we subsequently proceeded on testing the capability of MB cells to internalize large Dextran paricles (70 kDa Dextran – an established marker of macropinocytosis (Nakase, Hirose et al. 2009) upon pharmacological or genetic blockage of MAP4K4. Since macropinocytosis is generally

considered as a growth factor-dependent process (Haigler, Mckanna et al. 1979; Racoosin and Swanson 1989), we stimulated the cells with HGF. We found that the internalization of large dextran particles was decreased in MB cells with impaired MAP4K4 function, thus confirming our hypothesis that MAP4K4 is involved in the regulation of macropinocytosis.

Endocytic pathways including macropinocytosis have been linked in many studies to cell migration (Hattula, Furuhjelm et al. 2006; Beaumont, Hamilton et al. 2011; Rainero, Caswell et al. 2012; Nguyen, Grimm et al. 2017). Endocytosis plays key role during cell polarization, EMT transition and the formation of pro-migrating protrusions such as invadopodia and filopodia (Caswell, Spence et al. 2007; Poincloux, Lizarraga et al. 2009). One mechanism by which endocytosis controls cell migration is by trafficking adhesion molecules such as integrins, which is associated with the formation, turnover and recycling of focal adhesions and cell migration (reviewed in chapter 1.5.8 and 1.6.5).

5.5.2 Integrin turnover

Interestingly, MAP4K4 was also found to indirectly promote focal adhesion disassembly and integrin internalization (Yue and Wu 2014). Its *D. meanogaster* homologue (Misshapen) was identified as a key player during the morphogenesis of the Drosophila egg chamber by decreasing integrin levels at the basal surface, which facilitated detachment of each cell's trailing edge thus promoting migration (Lewellyn, Cetera et al. 2013). Yet another mechanism of MAP4K4-mediated focal adhesion disassembly was identified by Vitorino and colleagues, who showed that MAP4K4-ERM function in endothelial cells causes focal adhesion destablilization, which is necessary for efficient plasma membrane retraction during endothelial cell migration (Vitorino, Yeung et al. 2015).

We selected to study beta 1 integrin, not only because it was recently associated wih MAP4K4, but also because the dimers $\alpha_2\beta_1$ and $\alpha_1\beta_1$ consist the main receptors for type 1 collagen (Jokinen, Dadu et al. 2004), implying a crucial role for beta 1 integrin during cell invasion in collagen I, which has been used for the 3D cell motility assays and the integrin replating assays. We found that MAP4K4 depletion or inhibition with C29 caused reduced accumulation of activated integrin β_1 on endosomes in 2D. Similar results were acquired upon endocytosis inhibition using Dyngo 4a, suggesting that MAP4K4 capability to promote integrin internalization and signaling on endosomes depends on endocytosis. Integrins can transmit signals from endosomal locations during internalization, which promotes FAK phosphorylation and anoikis resistance, anchorage-independence and metastasis (Alanko, Mai et al. 2015). The same study reported MAP4K4 as a putative regulator of integrin β_1 on endosomal membranes, suggesting that endosomes act as platforms that support integrin signaling away from the plasma membrane. Our data on MAP4K4 mediated activation of integrins in endosomal compartments adds additional functional relevance to the proposed model of endosomal signaling. Consistent with a regulatory role of MAP4K4, we also detected reduced FAK phosphorylation in MAP4K4-depleted cells migrating inside collagen gels, further corroborating the critical role of MAP4K4 for functional integrin signaling during migration and invasion.

Interestingly, integrin activation consists a crucial factor for effcient 3D cell migration in collagen matrices (Mierke, Frey et al. 2010; Doyle, Carvajal et al. 2015). Using both an siRNA-mediated depletion approach and β_1 integrin blocking antibody, we deomonstrate that β_1 integrin function is essential for HGF-induced migration and invasion of MB cells in collagen as well. Hence, we subsequently attempted to test whether MAP4K4, being a mediator of β_1 integrin turnover, would affect integrin activation in 3D. Remarkably, stimulation of collagen-embedded DAOY cells with HGF led to the accumulation of activated β_1 integrin in cellular structures necessary for invasion (Fig. 5B, manuscript 2).

During tumor cell migration in 3D microenvironments, activated $\alpha_5\beta_1$ recycling promotes the extension of invasive pseudopodial structures, leading to increased invasiveness of the type associated with metastatic cancers (Caswell and Norman 2008; Muller, Caswell et al. 2009). Moreover, HGF and integrin cascades cooperate synergistically to induce FAK activation in an adhesion-dependent manner, leading to enhanced cell adhesion and motility (Beviglia and Kramer 1999). In our 3D assays we noticed the accumulation of activated β_1 integrin in cellular migrating protrusions especially upon HGF stimulation. However, this phenotype was abbrogated upon MAP4K4 depletion using the CRISPR/Cas9 system (Fig. 5B, manuscript 2), indicating that MAP4K4-dependent invasion in collagen relies on its ability to spatially coordinate

activated β_1 integrin. MAP4K4 appears to affect downstream integrin signaling as well, since immunoblot analysis revealed lower phopspho FAK (Tyr 397) levels in MAP4K4 depleted cells (Fig. 5E, manuscript 2). Furthermore, this finding corroborates the findings from Alanko and colleagues supporting that active FAK (Tyr 397) on endosomes results from integrin-ECM downstream signaling (Alanko, Mai et al. 2015).

5.5.3 c-Met turnover

Endosomes serve as signaling platforms not only for integrins but also for RTKs including c-Met (Masui, Castro et al. 1993). In fact, HGF stimulation can lead to clathrin-dependent internalization of c-Met, which leads to c-Met signaling form endosomal compartments and subsequent HGF-induced cell migration (Kermorgant and Scourzic 2005; Kermorgant and Parker 2005). c-Met has been shown to undergo Cbl–Grb2-dependent recycling causing thus aberrant activation of GTPase Rac1 and enhancing cell migration, anchorage-independent cell growth and in vivo tumorigenesis (Joffre, Barrow et al. 2011). Moreover, the integrin binding partner, RCP, was identified as a promoter of c-Met recycling in cells expressing oncogenic mutant p53 (Muller, Caswell et al. 2009). Depletion of RCP in mutant p53-expressing cells decreased the recycling of c-Met back to the plasma membrane, thereby attenuating ERK1/2 signaling and decreasing cell invasion and cell scattering. Moreover, in invasive, basal-like human breast cancer cell models, c-Met sustains Rac1 signaling, triggers membrane ruffling, cell migration and invasion (Menard, Parker et al. 2014). These data suggest that the imbalance between recycling and degradation in favour of continuous endosomal c-Met trafficking contributes to the maintenance of the activated state of c-Met, leading to pro-malignant signaling.

Based on the above observations, and since we have demonstrated that MAP4K4 promotes HGF-induced cell dissemination, we hypothesized that MAP4K4 mediates MB cell migration and invasion by promoting actin dynamics and turnover of c-Met receptors. We found that c-Met activation is impaired upon endocytosis inhibition and that MAP4K4 depletion phenocopies the reduced HGF-induced c-Met activation and phosphorylation of further downstream targets, suggesting that MAP4K4 mediates c-Met activation through endocytosis. These findings confirm previous observations pointing endocytosis as a mechanism of c-Met functions and establish for the first time MAP4K4 regulatory role during this process.

5.6 Conclusions

In summary, the findings provided in the present doctoral thesis emphasize MAP4K4 role in the regulation of Medulloblastoma dissemination. We provide evidence supporting MAP4K4 upregulated expression in Medulloblastoma tumors as well as its positive role towards Medulloblastoma dissemination. MAP4K4 controls actin dynamics and cytoskeletal rearrangements and facilitates cell migration. Additionally, MAP4K4 pro-migratory function was observed in response to IR, suggesting a potential contribution of MAP4K4 to IR-resistance of Medulloblastoma cells. Mechanistically, our data support a novel role for MAP4K4, whereby its control of cell migration is attributed to its function to regulate receptor endocytic turnover.

We propose a model whereby MAP4K4 upregulated expression enhances integrin signaling and RTK endocytic turnover. Endocytosed receptors will subsequently increase the activation of downstream targets which will eventually lead to enhanced cell dissemination. Increased dissemination levels could be caused on the one hand due to the increased integrin turnover that is responsible for FA disassembly and the generation of new FAs towards the leading edge. On the other hand increased integrin endosomal signaling provides enhanced survival and proliferative capabilities which allow such disseminating tumor cells to grow in new, foreign microenvironments. Importantly, it has been shown that aberrant recycling, activation and/or cell surface presentation of integrins can markedly alter the invasive behaviour of cancer cells. For instance, $\alpha_v\beta_6$ integrin increased recycling has been shown to promote oral cancer progression (Ramsay, Keppler et al. 2007). Furthermore, it has been demonstrated that inhibition of $\alpha_v\beta_3$ integrin promotes invasive cell migration by enhancing endosomal recycling of α5β1 integrin under the control of the Rab11 effector Rab coupling protein (RCP) (Rainero, Caswell et al. 2012). Interestingly, aberrant integrin signaling has also been

associated with mutated p53 expression. It is generally known that mutant p53 expression can promote invasion, loss of directionality of migration, and metastatic behavior (Vogelstein, Lane et al. 2000). Notably, these activities of p53 reflect enhanced integrin and epidermal growth factor receptor (EGFR) trafficking, which depends on Rab-coupling protein (RCP) and results in constitutive activation of EGFR/integrin signaling.

Another aspect of MAP4K4-mediated pro-invasive aspects includes the endocytosis of c-Met. Our findings suggest that MAP4K4 could mediate HGF-induced cell motility through the constant endocytosis of c-Met receptor. Aberrant c-Met endocytosis and recycling has been identified as a pro-invasive mechanism (Joffre, Barrow et al. 2011). Therefore, although further experiments need to be performed in order to list the endocytic pathways that are dependent on MAP4K4, our data suggest its role as a promoter of endocytosis and that this function is able to elicit a migratory behavior of cancer cells. This migratory behavior can be generated through the formation of actin-rich migratory cell protrusions at the leading edge of the migrating cells and contribute to the penetration of 3D matrices.

Furthermore, this endocytosis-associated pro-migratory function of MAP4K4 could be responsible for the abovely described IR-induced MB cell dissemination, either through increased integrin expression and endosomal signaling, or upregulated c-Met recycling, though further experiments will be needed to address this hypothesis.

Cumulatively, in the present study we introduce MAP4K4 regulatory role in membrane receptor turnover as a prominent cause of Medulloblastoma cell infiltration. MAP4K4 can be pharmacologically targeted and therefore contribute to novel specific Medulloblastoma therapeutic approaches.

7. References

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8. Abbreviations

2D	Two-Dimensional
3D	Three-Dimensional
aa	amino acid
AKT	Protein Kinase B
Arp2/3	Actin-Related Proteins 2 and 3
bFGF	Basic Fibroblast Growth Factor
BMPs	Bone Morphogenic Proteins
c-Met	c-Mesenchymal Epithelial Transition
CAF	Carcinoma Associated Fibroblasts
cdc42	Cell Division Cycle 42
CDR	Circular Dorsal Ruffle
CLASPs	Cytoplasmic Linker Associated Proteins
CLIC3	Chloride Intracellular Channel Protein 3
CNH	Citron Homology Domain
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CT	Chemotherapy
CTNNB1	beta catenin 1
Dock	Dedicator of cytokinesis
ECM	Extracellular Matrix
EEA1	Early Endosome Antigen 1
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGL	External Granule layer
ELMO1	Engulfment and cell motility protein 1
EMT	Epithelial to Mesenchymal Transition
ER+	estrogen receptor–positive
Erk 1/2	Extracellular Signal-Regulated Kinases 1 and 2
ERM	Ezrin, Radixin and Moesin
FA	Focal Adhesion
FAK	Focal Adhesion Kinase
FGFR	FGF Receptor
FMNL2	Formin-Like 2-Actin Nucleation and Assembly Factor
FN	Fibronectin
GEF	Guanine nucleotide exchange factor
GM-CSF	Granulocyte - monocyte Colony Stimulating Factor
GNP	Granule Neuron Precursors
Grb2	Growth factor receptor-bound protein 2
HGF	Hepatocyte Growth Factor
IF	Immunofluorescence
IGF	Insulin-like Growth Factor
IGL	Internal Granule Layer
IR	Ionizing radiation
ITGB1	Integrin beta 1
JNK	c-Jun N-terminal kinase
K/D	Kinase/Dead
L1-CAM	L1-Cell Adhesion Molecule
LC/A MB	Large Cell Anaplastic Medulloblastoma
MAP4K4	Mitogen-Activated Protein Kinase Kinase Kinase Kinase 4
MAPK	Mitogen ActivatedProtein Kinase
MB	Medulloblastoma
MBEN	Medulloblastoma with Extensive Nodularity

Med PDX	Medulloblastoma Patient – Derived Xenografts
MMPs	Matrix Metalloproteinases
MT1-MMP	Membrane-Type 1 Matrix Metalloprotease
mTOR	Mammalian Target Of Rapamycin
N0	Lymph Node–Negative
NFκB	Nuclear Factor kappa – light – chain – enhancer of activated B cells
PAK	p21 Activated Kinase
PDGF	Platelet-Derived Growth Factor
PDX	Patient Derived Xenografts
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKB	Protein Kinase B
PKL	Protein Kinase-Like
PLCγ	phospholipase Cγ
PTCH1	Patched 1
PYK2	Proline rich Tyrosine Kinase 2
q-PCR	quantitative-Polymerase Chain Reaction
Rac1	Ras – related C3 botulinum toxin substrate 1
RASA1	Ras GTPase-Activating Protein 1
RCP	Rab-Coupling Protein
RhoA	Ras homolog gene family, member A
RhoC	Ras homolog gene family, member C
ROCK	Rho associated protein kinase
RT	Radiation Therapy
RTK	Receptor Tyrosine Kinase
SH2	Src - homology 2
SH3	Src - homology 3
SHH	Sonic Hedgehog
SIA	Spheroid Invasion assay
SMO	Smoothened
Sos	Son of Sevenless
Src kinase	Proto - oncogene tyrosine - protein kinase Src
STAT3	Signal Transducer and Activator of Transcription 3
TGF - β	Transforming Growth Factor - β
TNF - α	Tumour Necrosis Factor - alpha
TPC	Two-Pore Channel
TRAF	Tumor Necrosis Factor Receptor Associated Factor
uPA	Urokinase Plasminogen Activator
URL	Upper Rhombic Lip
VEGF	Vascular Endothelial Growth Factor
VZ	Ventricular Zone
WB/IB	Western Blot/Immunoblot
WHO	World Health Organization
WNT	Wingless
wt	wild-type
YAP1	Yes Associated Protein 1
β-PIX	Rho guanine nucleotide exchange factor 7

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Ἰθάκη

Σὰ βγεῖς στὸν πηγαιμὸ γιὰ τὴν Ἰθάκη,
νὰ εὔχεσαι νά 'ναι μακρὺς ὁ δρόμος,
γεμάτος περιπέτειες, γεμάτος γνώσεις.

Τοὺς Λαιστρυγόνας καὶ τοὺς Κύκλωπας,
τὸν θυμωμένο Ποσειδῶνα μὴ φοβᾶσαι,
τέτοια στὸν δρόμο σου ποτέ σου δὲν θὰ βρεῖς,
ἂν μέν' ἡ σκέψις σου ὑψηλή, ἂν ἐκλεκτὴ
συγκίνησις τὸ πνεῦμα καὶ τὸ σῶμα σου ἀγγίζει.

Τοὺς Λαιστρυγόνας καὶ τοὺς Κύκλωπας,
τὸν ἄγριο Ποσειδῶνα δὲν θὰ συναντήσεις,
ἂν δὲν τοὺς κουβανεῖς μὲς στὴν ψυχή σου,
ἂν ἡ ψυχή σου δὲν τοὺς στήνει ἐμπρός σου.

Νὰ εὔχεσαι νά 'ναι μακρὺς ὁ δρόμος.
Πολλὰ τὰ καλοκαιρινὰ πρωινὰ νὰ εἶναι
ποῦ μὲ τί εὐχαρίστηση, μὲ τί χαρὰ
θὰ μπαίνεις σὲ λιμένας πρωτοειδωμένους.

Νὰ σταματήσεις σ' ἐμπορεῖα Φοινικικά,
καὶ τὲς καλὲς πραγμάτειες ν' ἀποκτήσεις,
σεντέφια καὶ κοράλλια, κεχριμπάρια κ' ἔβενους,
καὶ ἡδονικὰ μυρωδικὰ κάθε λογῆς,
ὅσο μπορεῖς πιὸ ἄφθονα ἡδονικὰ μυρωδικά.

Σὲ πόλεις Αἰγυπτιακὲς πολλὲς νὰ πᾷς,
νὰ μάθεις καὶ νὰ μάθεις ἀπ' τοὺς σπουδασμένους.
Πάντα στὸ νοῦ σου νά 'χεις τὴν Ἰθάκη.
Τὸ φθάσιμον ἐκεῖ εἶν' ὁ προορισμός σου.

Ἀλλὰ μὴ βιάζεις τὸ ταξίδι διόλου.
Καλλίτερα χρόνια πολλὰ νὰ διαρκέσει.
Καὶ γέρος πιὰ ν' ἀράξεις στὸ νησί,
πλούσιος μὲ ὅσα κέρδισες στὸν δρόμο,
μὴ προσδοκώντας πλούτη νὰ σὲ δώσει ἡ Ἰθάκη.

Ἡ Ἰθάκη σ' ἔδωσε τ' ὡραῖο ταξίδι.
Χωρὶς αὐτὴν δὲν θά 'βγαινες στὸν δρόμο.
Ἄλλα δὲν ἔχει νὰ σὲ δώσει πιά.

Κι ἂν πτωχικὴ τὴν βρεῖς, ἡ Ἰθάκη δὲν σὲ γέλασε.
Ἔτσι σοφὸς ποὺ ἔγινες, μὲ τόση πεῖρα,
ἤδη θὰ τὸ κατάλαβες οἱ Ἰθάκες τὶ σημαίνουν.

Κωνσταντίνος Π. Καβάφης

Ithaka

Once you set out for Ithaka
hope your road to be long,
full of adventures, full of knowledge.

Don't be afraid of the Laistrygonians and the Cyclops,
the angry Poseidon
you'll never find them on your way
if you keep your thoughts high,
if rare excitement touches your spirit and your body.

You won't meet the Laistrygonians and the Cyclops,
the wild Poseidon
unless you bring them along inside your soul,
unless your soul puts them in front of you.

Hope your road to be long
may there be many summer mornings
when you'll enter with pleasure, with joy,
the harbours you've seen for the first time

Stop in Phoenician trading stations
and get the good wares
pearls and corals, ambers and ebony,
and sensual herbs of every kind
as many sensual herbs as you can

Go to many Egyptian cities
to study and learn from the educated ones
keep Ithaka always in your mind
your arrival there is your destiny

But don't rush the journey at all
it better lasts for many years,
and then when you're old to stay on the island,
wealthy with all you've gained on the way
without expecting Ithaka to make you rich.

Ithaka gave you the beautiful journey.
without her you wouldn't have set out
there's nothing else to give you anymore

And if you find her poor, Ithaka hasn't fooled you.
now that you became wise with so much experience
you should have already understood what Ithakas mean

Constantine P. Cavafy

10. Auxiliary

10.1 Teaching hours

University of Zurich[UZH]

Division of Biology / Fachbereich Biologie

Summary of teaching activities

This form[1] is to be completed and signed by the PhD student at the **end of the doctorate**, endorsed by the responsible professor[2] (rP), and submitted to the Office of the Dean of Studies when registering for the doctoral examination.

Please send a copy to: University of Zurich, Academic Support Office ASO ("Studienkoordination des Fachbereichs Biologie"), Division of Biology Y13-J-01, Winterthurerstrasse 190, 8057 Zürich studienkoordination.biologie@uzh.ch

Name of PhD candidate:	Dimitra Tripolitsioti
Responsible professor	Martin Baumgartner
Start date of PhD	June 1st 2013
End date of PhD	~~May~~ 30th 2017

Summary of teaching activities

Semester	Teaching activity in Module Number … (if applicable)	Hours
HS 2013	Blockkurs B10319	25
HS 2014	Blockkurs B10319	25
HS 2014	Master student	50
	TOTAL	100

Signatures

Date: 4/4/17 PhD Candidate .....

Date 4.4.2017 Responsible professor .....

(See page 2 for descriptions of which activities can and cannot count towards the teaching requirement)

[1] Download under www.biologie.uzh.ch/Studium/Studiengang/Doktorat.html

[2] The rP is an MNF faculty member or somebody with "Promotionsrecht" at the MNF. The rP normally heads the PhD committee.

University of Zurich[UZH]

Which activities count towards teaching hours?

- Actual time spent teaching students in laboratory practicals, classroom sessions, lectures, exercise classes, fieldwork etc.
- Preparation
- Correcting students' reports
- Supervising /coaching in semester projects, summer schools, research practicals
- Marking exams
- Overseeing exams
- Master's thesis supervision[3]
- Instructor in the "Life Science Zurich – Learning Center (LSLC)"[4]

Which activities do NOT count as teaching?

- Journal clubs
- PhD student seminars
- Modules primarily for PhD students and where they acquire credit points
- Introducing new PhD students to the laboratory equipment and procedures of the research group
- Progress reports

In general: all those activities in which PhD students in purely research institutes would be involved (such as, e.g., Max-Planck Institutes)

[3] The Division Council recommends counting between **0 and 50 hours per Master's thesis**. The responsible professor decides on the final allocation of hours depending on the extent of scientific benefit that the PhD student gains from the supervision of the Master student (the higher the benefit, the fewer the hours). Good supervision of a Master student incorporates important components of teaching and learning: **the allocation of 0 hours should not be practiced in such cases**.

It is not recommended that PhD students fulfill their teaching requirement exclusively with supervision of Master students.

[4] PhD students interested in this teaching opportunity should first consult the paper "Rules for the partial fulfillment of the MNF teaching requirement within the LSLC" in which they will find all the details

10.2 List of credit points

University of Zurich[UZH] | ETH zürich

List of activities

Student	Dimitra Tripolitsioti
Matriculation number	13-762-760
Faculty/Department	Faculty of Science (UZH) (PhD student)

Title# *Module number*	*Category/* *Type*	*Lecturer/* *Offered by*	*Location/* *End date*	*Credits/* *Hours*
1st-year-presentations	Compulsory activity Transferable skills	Susanna Bachmann PhD program	Zurich 2013-10-18	1.00 25 h
NGS Sequencing BIO680	Core elective activity Methods	Lucy Poveda & Lennart Opitz LSZGS	Zurich 2013-10-18	2.00 40 h
Model Organisms in Developmental Biology BIO663	Core elective activity Subject-specific matters	Alex Hajnal PhD program	Zurich 2013-09-04	1.00 27 h
Scientific Writing	Compulsory activity Transferable skills	Sabine Schrimpf LSZGS	Zurich 2015-03-24	1.00 25 h
11th MLS retreat	Compulsory activity Research seminars	Retreat Organizing Committee (ROC) - Susanna Bachmann PhD program	Rigi Kaltbad 2014-08-30	1.00 25 h
First steps into teaching and Learning in Biology	Core elective activity Transferable skills	Dr. P. Alean-Kirckpatrick UZH	Zurich 2014-01-13	1.00 2 h
Practical Course in Advanced Microscopy	Elective activity Research seminars	Gabor Csucs, Andres Kaech, Roger Wepf, Urs Ziegler PhD program	Zurich 2015-05-26	2.00 25 h
Germal Language Certificate	Elective activity Transferable skills	lic. phil. Anja Gredig PhD program	Zurich 2014-12-18	2.00 20 h
12th MLS retreat	Core elective activity Research seminars	Retreat Organizing Committee (ROC) - Susanna Bachmann PhD program	Engelberg 2015-08-29	1.00 25 h
Course on Ethics in Science	Compulsory activity Transferable skills	Gerd Folkers PhD program	Zurich 2015-10-20	1.00 25 h
13th MLS retreat	Elective activity Summer school / Retreat	Retreat Organizing Committee (ROC) - Susanna Bachmann PhD program	Kiental 2016-09-03	1.00 25 h
Total				14.00 264 h

Signature of Official Supervisor: G. Bargmann

Date: 4.4.2017

10.3 Curriculum vitae

Dimitra TRIPOLITSIOTI

Biology Scientist, PhD candidate

07.08.1987
Greek, Permit B
Forchstrasse 158 • 8032, Zurich • Switzerland
0789415629 • dimitratripol@gmail.com•
www.linkedin.com/in/dimitra-tripolitsioti

Employed as a PhD student at UZH since June 2013

EDUCATION

06/2013 - 05/2017 **Doctoral studies in the University of Zurich**, Molecular Life Sciences Graduate Program.

Doctoral thesis performed in the Institute of Experimental Infectiology and Cancer Research of the University Children's Hospital Zurich.

Specialization in Cell, Molecular & Cancer Biology.

10/2010 - 04/2013 **Master Degree in Molecular Biotechnology**, University of Patras, Greece.

Specialization in Cell, Molecular and Cancer Biology.

Master thesis title: *HARP growth factor activates indirectly ALK membrane receptor*

09/2005 - 09/2010 **Bachelor Degree in General Biology**, University of Patras, Greece.

05/2002 – 09/2005 **Graduation from the 2nd High school** of Patras, Greece.

Specialization in Natural Sciences (2002 – 2005).

Marks obtained: 18.8/20 – Distinstion/Excellent

Coursework: Mathematics, Physics, Chemistry, Biology

PROFFESSIONAL EXPERIENCE/TRAINING

06/2013 - 05/2017 Researcher in the Neurooncology group of the Children's Hospital Zurich.

- Focus on the molecular mechanisms of metastasis in Medulloblastoma, the most common malignant brain tumor in childhood.

09/2011 - 04/2013 Teaching assistant in the University of Patras, Greece.

- Cell & Molecular Biology in Bachelor's laboratory classes

05/2011 – 08/2011 Internship in CRRET CNRS, Paris-Est Creteil, Paris, France.

- Protein purification methods

- Cancer cell response to anti-metastatic compounds

EXTRA-CURRICULAR

06/2013 - 06/2015 Member of the marketing team of ETH-GETHIRED (https:// eth-gethired.ch/)

06/2014 - 06/2015 Member of the board committee of the Hellenic Student Association of Zurich

CERTIFICATES / COURSES during PhD studies

- Project management in research
- Scientific writing in research
- Teaching in laboratory classes, teaching in english, teaching & learning in biology

TECHNICAL SKILLS

- Expertise in advanced molecular and cell biology techniques (Cell culture, qPCR, Western Blot, Immunoprecipitation, ELISA)
- Advanced cell imaging qualifications (Experience with Leica & ZEISS equipment, Imaris and ImageJ)
- ECDL core certificate (Fluency in Microsoft Office packages)
- Genomic platforms handling

QUALIFICATIONS – TRANSFERABLE SKILLS

- Ability to present and organize large amounts of information in a clear manner.
- Analysis of complex data and presentation of emerging conclusions and concepts.
- Problem-solving skills
- Ability to communicate complex ideas effectively in large formats
- Ability to plan a project and deliver it to agreed timelines
- Capability to work as part of a team, as well as with minimum supervision
- Ability to communicate effectively to a range of audiences
- Initiative and self-reliance
- Negotiation skills
- Event planning skills

LANGUAGES

Greek: Native
English: Professional working proficiency
French: Limited working proficiency
German: Elementary working proficiency
Italian: Basic

SCIENTIFIC PUBLICATIONS

- The Ser/Thr Kinase MAP4K4 Controls Pro-Metastatic Cell Functions.

Dimitra Tripolitsioti, Michael A Grotzer, Martin Baumgartner (Carcinogenesis & Mutagenesis, in press).

- A novel approach to investigate cellular and molecular determinants of brain tissue infiltration in medulloblastoma.
  Anuja Neve, Karthiga Santhana Kumar, **Dimitra Tripolitsioti**, Michael A Grotzer, Martin Baumgartner (Scientific Reports, Nature Publishing Group, in press).
- The Ser/Thr kinase MAP4K4 drives c-Met-induced motility and invasiveness in a cell-based model of SHH medulloblastoma.
  Karthiga Santhana Kumar, **Dimitra Tripolitsioti**, Min Ma, Jasmin Grählert, Katja B Egli, Giulio Fiaschetti, Tarek Shalaby, Michael A Grotzer, Martin Baumgartner (Springerplus, 2015).

CONFERENCES

- EMBO, 2016 in Cavtat, Croatia
  Poster: MAP4K4 control of cell migration in Medulloblastoma

PUBLIC COMMUNICATIONS

Oral Presentations:

- Jan 2017, Jan 2015: Cytomeet, Bern, Switzerland
- May 2016, Nov 2014: Kolloquium of applied cancer research, Zürich, Switzerland

Poster Presentations:

- Jan 2017, Jan 2015: C.B. Brupbacher symposium, Zürich, Switzerland
- Aug. 2014, 2015 & 2016: Children's research centre retreat, Zürich, Switzerland

GRANTS

- Travel Grant from the Molecular Life Sciences PhD program for EMBO 2016, 700 CHF

REFERENCES

Available upon request